ICCVAM Test Method Evaluation Report: Usefulness and Limitations of the Murine Local Lymph Node Assay for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans

Interagency Coordinating Committee on the Validation of Alternative Methods

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

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

2011

NIH Publication Number 11-7709

National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

This document is available electronically at
http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/TMER.htm

When referencing this document, please cite as follows:
ICCVAM. 2011. ICCVAM Test Method Evaluation Report: Usefulness and Limitations of the Murine Local Lymph Node Assay for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans. NIH Publication No. 11-7709. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

Table of Contents

List of Tables ..... **vi**

List of Figures ..... **vii**

List of Abbreviations and Acronyms ..... **viii**

Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives ..... **ix**

Acknowledgements ..... **x**

Preface ..... **xv**

Executive Summary ..... **xvii**

1.0 Introduction **1**

1.1 Background Information on the Murine Local Lymph Node Assay 1

1.2 ICCVAM, NICEATM, and Interagency Immunotoxicity Working Group 1

1.3 LLNA Background Review Document 1

1.4 Peer Review Panel 2

1.5 Scientific Advisory Committee on Alternative Toxicological Methods 2

1.6 Final ICCVAM Test Method Recommendations and Final Background Review Document 3

2.0 ICCVAM Recommendations: Usefulness and Limitations of the LLNA for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans, Test Method Protocol, and Future Studies **4**

2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations 4

2.2 ICCVAM Recommendations: Test Method Protocol 5

2.3 ICCVAM Recommendations: Future Studies 5

3.0 Validation Status for Use of the LLNA to Determine Skin Sensitization Potency Categories **6**

3.1 Test Method Description 6

3.1.1 General Test Method Procedures 6

3.2 Validation Database 6

3.3 Reference Test Method Data 8

3.4 Test Method Accuracy 8

3.4.1 DSA_{05} and EC3 Values 8

3.4.2 LLNA Classification of Strong and Other Than Strong Sensitizers in Humans 10

3.5 Test Method Reliability 13

3.5.1 Intra- and Interlaboratory Variability 13

3.5.2 Influence of LLNA Vehicle 13

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement 14

4.0 ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments 15

4.1 ICCVAM Consideration of Independent Peer Review Panel Report 15

4.1.1 Comments on Draft ICCVAM Recommendations: Test Method Usefulness and Limitations 15

4.1.2 Comments on Draft ICCVAM Recommendations: Test Method Protocol 15

4.1.3 Comments on Revised Draft ICCVAM Recommendations: Future Studies 16

4.2 ICCVAM Consideration of Public and SACATM Comments 16

4.2.1 Public Comments in Response to 72 FR 27815 (May 17, 2007): The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data 17

4.2.2 Public Comments in Response to 72 FR 52130 (September 12, 2007): Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments 19

4.2.3 Public Comments in Response to 73 FR 1360 (January 8, 2008): Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments 19

4.2.4 Public Comments in Response to 73 FR 25754 (May 7, 2008): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) 21

4.2.5 Public Comments in Response to 73 FR 29136 (May 20, 2008): Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments 22

4.2.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008 22

5.0 References 23

Appendix A Timeline for ICCVAM Evaluation on Usefulness and Limitations of the Murine Local Lymph Node Assay for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans A-1

Appendix B Updated ICCVAM-Recommended Protocol: The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products B-1

Appendix C Final Background Review Document: Use of the Murine Local Lymph Node Assay for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans C-1

Appendix D Independent Scientific Peer Review Panel Assessment D-1

D1 Summary Minutes from the Independent Scientific Peer Review Panel Meeting on March 4-6, 2008 ..... D-3

D2 Peer Review Panel Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products D-33

Appendix E GHS Materials on Skin Sensitization Potency E-1

E1 Meetings of the Sub-Committee of Experts on the Globally Harmonized System of Classification and Labelling of Chemicals Relevant to Subcategorization of Skin Sensitizers E-3

E2 ST/SG/AC.10/C.4.2006/16, Strong versus Weak Sensitizers E-9

E3 UN/SCEGHS/12/INF.16 (OECD), Progress Report on OECD Work Since the Last Sub-Committee Meeting E-23

E4 UN/SCEGHS/15/INF.14 (OECD), Proposal for Revising Chapter 3.4 with Respect to Strong versus Weak Sensitizers: Explanatory Notes and Revised Chapter with Visible Changes E-27

E5 ST/SG/AC.10/C.4/2008/18 (Secretariat), Revision of Chapter 3.4 with Respect to Strong versus Weak Sensitizers E-43

E6 ST/SG/AC.10/C.4/2008/18/Add.1 (Secretariat), Revision of Chapter 3.4 with Respect to Strong versus Weak Sensitizers, Addendum E-55

E7 UN/SCEGHS/16/INF.3 (Secretariat), Section 3.4.2 of Chapter 3.4 ..... E-63

Appendix F *Federal Register* Notices and Public Comments F-1

F1 *Federal Register* Notices F-3

F2 Public Comments Received in Response to *Federal Register* Notices F-17

F3 SACATM Comments: SACATM Meeting, June 18-19, 2008 ..... F-93

Appendix G Relevant Skin Sensitization Regulations and Testing Guidelines G-1

G1 Table of Relevant Skin Sensitization Test Regulations G-3

G2 EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization (March 2003) G-7

G3 International Organization for Standardization – ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Skin Sensitization (July 2010) G-25

G4 OECD Test Guideline 429: Skin Sensitization – Local Lymph Node Assay (Adopted July 2010) G-27

G5 OECD Test Guideline 406: Skin Sensitisation (Adopted July 1992) G-49

List of Tables

Table 3-1 Chemical Classes Represented in the LLNA Potency Database 7

Table 3-2 Distribution of 136 Substances for Classification Rate Analyses.....9

Table 3-3 Concordance of LLNA and Human Data for Strong Sensitizer, Other Sensitizer, and Nonsensitizer Categories for 136 Substances at Selected LLNA EC3 Values 11

Table 3-4 Classification Rates for the Prediction of Human Potency Categories by Selected LLNA EC3 Cutoff Values for 136 Substances 12

Table 4-1 Opportunities for Public Comments 17

List of Figures

Figure 3-1 LLNA EC3 and Human Results by GHS Potency Category for 136 Substances 9

Figure 3-2 Classification Rates for the LLNA EC3 Prediction of 27 Strong Human Sensitizers..... 12

List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
BRD	Background review document
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
DSA	Dose per skin area
DSA_{05}	Induction dose per skin area, in $\mu g/cm^2$, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population
EC3	Estimated concentration of a substance expected to produce a stimulation index of 3, the threshold value for a substance to be considered a sensitizer in the LLNA
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FR	*Federal Register*
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
HMT	Human maximization test
HRIPT	Human repeat-insult patch test
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
LLNA	Murine local lymph node assay
LOEL	Lowest observed effect level
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
n	Number
NOEL	No observed effect level
OECD	Organisation for Economic Co-operation and Development
R^2	Coefficient of determination
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index

Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives

Agency for Toxic Substances and Disease Registry
* Moiz Mumtaz, Ph.D.
Bruce Fowler, Ph.D.
Edward Murray, Ph.D.
Eric Sampson, Ph.D.

Consumer Product Safety Commission
* Marilyn L. Wind, Ph.D. (Chair, to July 2010)
+ Kristina Hatlelid, Ph.D.
Joanna Matheson, Ph.D.

Department of Agriculture
* Jodie Kulpa-Eddy, D.V.M. (Acting Chair)
+ Elizabeth Goldentyer, D.V.M.

Department of Defense
* David Honey, Ph.D.
* Robert E. Foster, Ph.D. (to Aug. 2010)
+ Patty Decot
Harry Salem, Ph.D. (to Aug. 2010)
Terry Besch, D.V.M., M.S., DACLAM, DACVPM
Peter J. Schultheiss, D.V.M., DACLAM (to Aug. 2010)

Department of Energy
* Michael Kuperberg, Ph.D.
+ Marvin Stodolsky, Ph.D.

Department of the Interior
* Barnett A. Rattner, Ph.D.
+ Sarah Gerould, Ph.D. (to Feb. 2009)

Department of Transportation
* George Cushmac, Ph.D.
+ Steve Hwang, Ph.D.

Environmental Protection Agency
Office of Pesticide Programs
* John R. "Jack" Fowle III, Ph.D., DABT
+ Vicki Dellarco, Ph.D.
+ Tina Levine, Ph.D.
Deborah McCall
Christine Augustyniak, Ph.D. (*U.S. Coordinator, OECD Test Guidelines Program)*
Office of Pollution Prevention and Toxics
Jerry Smrchek, Ph.D. (*U.S. Coordinator, OECD Test Guidelines Program,* to July 2009*)*
Office of Research and Development
Suzanne McMaster, Ph.D. (to Dec. 2008)
Julian Preston, Ph.D. (to July 2009)
Stephanie Padilla, Ph.D. (to July 2009)
Office of Science Coordination and Policy
Karen Hamernik, Ph.D. (to July 2009)

Food and Drug Administration
Office of the Commissioner
* Suzanne Fitzpatrick, Ph.D., DABT
Center for Biologics Evaluation and Research
Richard McFarland, Ph.D., M.D.
Ying Huang, Ph.D.
Center for Devices and Radiological Health
Melvin E. Stratmeyer, Ph.D. (to May 2010)
Vasant G. Malshet, Ph.D., DABT
Center for Drug Evaluation and Research
+ Abigail C. Jacobs, Ph.D.
Paul C. Brown, Ph.D.
Center for Food Safety and Applied Nutrition
David G. Hattan, Ph.D.
Neil Wilcox, D.V.M., M.P.H.
Robert L. Bronaugh, Ph.D. (to May 2010)
Center for Veterinary Medicine
Devaraya Jagannath, Ph.D.
M. Cecilia Aguila, D.V.M.
National Center for Toxicological Research
Paul Howard, Ph.D.
Donna Mendrick, Ph.D.
William T. Allaben, Ph.D. (to Jan. 2009)
Office of Regulatory Affairs
Lawrence D'Hoostelaere, Ph.D.

National Cancer Institute
* T. Kevin Howcroft, Ph.D.
Chand Khanna, D.V.M., Ph.D.
Alan Poland, M.D. (to Oct. 2008)

National Institute of Environmental Health Sciences
* William S. Stokes, D.V.M., DACLAM
+ Warren Casey, Ph.D., DABT
Raymond R. Tice, Ph.D.
Rajendra S. Chhabra, Ph.D., DABT
Jerrold J. Heindel, Ph.D.

National Institute for Occupational Safety and Health
* Paul Nicolaysen, V.M.D.

National Institutes of Health
* Margaret D. Snyder, Ph.D.

National Library of Medicine
* Pertti (Bert) Hakkinen, Ph.D.
+ Jeanne Goshorn, M.S.

Occupational Safety and Health Administration
* Surender Ahir, Ph.D.

* Principal agency representative
+ Alternate principal agency representative

Acknowledgements

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Interagency Immunotoxicity Working Group (IWG)

U.S. Consumer Product Safety Commission
Joanna Matheson, Ph.D. (IWG Co-chair)
Marilyn L. Wind, Ph.D. (to July 2010)

U.S. Environmental Protection Agency
Office of Pesticide Programs
Jonathan Chen, Ph.D.
Masih Hashim, D.V.M., Ph.D.
Marianne Lewis
Deborah McCall
Timothy McMahon, Ph.D.
John Redden, M.S.
Jenny Tao, Ph.D.
Office of Pollution Prevention and Toxics
Elizabeth Margosches, Ph.D.
Ronald Ward, Ph.D.
Office of Research and Development
Marsha Ward, Ph.D.
Office of Science Coordination and Policy
Karen Hamernik, Ph.D.

U.S. Food and Drug Administration
Center for Devices and Radiological Health
Vasant G. Malshet, Ph.D., DABT
Jeffrey Toy, Ph.D.
Center for Drug Evaluation and Research
Ruth Barratt, Ph.D., D.V.M.
Paul C. Brown, Ph.D.
Abigail C. Jacobs, Ph.D. (IWG Co-chair)
Jiaqin Yao, Ph.D.
Office of Science and Health Coordination
Suzanne Fitzpatrick, Ph.D., DABT

National Institute of Environmental Health Sciences
Warren Casey, Ph.D., DABT
Dori Germolec, Ph.D.
William S. Stokes, D.V.M., DACLAM

National Institute for Occupational Safety and Health
B. Jean Meade, D.V.M., Ph.D.
Paul D. Siegel, Ph.D.

National Library of Medicine
Pertti (Bert) Hakkinen, Ph.D.

European Centre for the Validation of Alternative Methods – Liaison
Silvia Casati, Ph.D.
Alexandre Angers, Ph.D.

Japanese Center for the Validation of Alternative Methods – Liaison
Hajime Kojima, Ph.D.

Murine Local Lymph Node Assay
Independent Scientific Peer Review Panel
(March 4-6, 2008)

Michael Luster, Ph.D. (Panel Chair)
Senior Consultant to the National Institute for Occupational Safety and Health
Health Effects Laboratory
Morgantown, WV

Nathalie Alépée, Ph.D.
Scientific Coordinator on Alternatives Methods in Life Science
L'Oréal Research and Development
Aulnay sous Bois, France

Anne Marie Api, Ph.D.
Vice President, Human Health Sciences
Research Institute for Fragrance Materials
Woodcliff Lake, NJ

Nancy Flournoy, M.S., Ph.D.
Professor and Chair
Department of Mathematics and Statistics
University of Missouri – Columbia
Columbia, MO

Thomas Gebel, Ph.D.
Regulatory Toxicologist
Federal Institute for Occupational Safety & Health
Dortmund, Germany

Sidney Green, Ph.D.[1]
Graduate Professor
Howard University
Washington, DC

Kim Headrick, B.Admin., B.Sc.
International Harmonization and Senior Policy Advisor
Policy and Programme Service Office
Health Canada
Ottawa, Ontario, Canada

Dagmar Jírová, M.D., Ph.D.
Toxicologist, Research Manager
Head of Reference Center for Cosmetics and Reference Laboratory for Experimental Immunotoxicology
National Institute of Public Health
Prague, Czech Republic

David Lovell, Ph.D., FIBiol, CStat, CBiol
Reader in Medical Statistics
Postgraduate Medical School
University of Surrey
Guildford, Surrey, U.K.

Howard Maibach, M.D.
Professor, Department of Dermatology
University of California – San Francisco
San Francisco, CA

James McDougal, Ph.D.
Professor and Director of Toxicology Research
Department of Pharmacology and Toxicology
Boonshoft School of Medicine
Wright State University
Dayton, OH

Michael Olson, Ph.D., A.T.S.
Director of Occupational Toxicology
Corporate Environment, Health and Safety
GlaxoSmithKline
Research Triangle Park, NC

Raymond Pieters, Ph.D.
Associate Professor
Immunotoxicology Group Leader
Institute for Risk Assessment Sciences
Utrecht University
Utrecht, The Netherlands

Jean Regal, Ph.D.
Professor, Department of Pharmacology
University of Minnesota Medical School
Duluth, MN

Jonathan Richmond, MB ChB, FRCSEd[1]
Head, Animals Scientific Procedures Division
Home Office
London, U.K.

Peter Theran, V.M.D.
Consultant
Massachusetts Society for the Prevention of Cruelty to Animals
Novato, CA

Stephen Ullrich, Ph.D.
Professor of Immunology
Graduate School of Biomedical Sciences
University of Texas
M.D. Anderson Cancer Center – Houston
Houston, TX

Michael Woolhiser, Ph.D.
Science and Technology Leader
Toxicology and Environmental Research and Consulting
The Dow Chemical Company
Midland, MI

Takahiko Yoshida, M.D., Ph.D.
Professor, Department of Health Science
Asahikawa Medical College
Hokkaido, Japan

[1] Drs. Green and Richmond were unable to attend the public meeting on March 4-6, 2008. However, they were involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

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

National Institute of Environmental Health Sciences

William S. Stokes, D.V.M., DACLAM
Director

Warren Casey, Ph.D., DABT
Deputy Director

Deborah McCarley
Special Assistant; Assistant Project Officer

NICEATM Support Contract Staff (Integrated Laboratory Systems [ILS], Inc.)

David Allen, Ph.D.
Thomas Burns, M.S.
Linda Litchfield
Steven Morefield, M.D.
Michael Paris
Eleni Salicru, Ph.D.
Catherine Sprankle
Frank Stack
Judy Strickland, Ph.D., DABT
Linda Wilson

Statistical Consultant for ILS, Inc.

Joseph Haseman, Ph.D.

Other Acknowledgements

ICCVAM and NICEATM gratefully acknowledge the following individuals and institutions for submitting data to NICEATM for the evaluation of the use of the LLNA to determine skin sensitization potency categories.

Anne Marie Api, Ph.D.
Research Institute for Fragrance Materials
Woodlake, NJ

David Basketter, Ph.D.[1]
Unilever Safety and Environmental Assurance Centre
Sharnbrook, U.K.

Phil Botham, Ph.D.
European Crop Protection Association
Brussels, Belgium

Eric Debruyne, Ph.D.
Bayer CropScience SA, Sophia Antipolis
Cedex, France

G. Frank Gerberick, Ph.D.
The Procter & Gamble Company
Cincinnati, OH

Dori Germolec, Ph.D.
National Toxicology Program
Research Triangle Park, NC

Ian Kimber, Ph.D.[2]
Syngenta Central Toxicology Laboratory
Macclesfield, U.K.

Heidi Ott
Federal Institute for Occupational Safety and Health
Dortmund, Germany

Kirill Skirda, Ph.D.
TNO Quality of Life
Delft, The Netherlands

Peter Ungeheuer, Ph.D.
European Federation for Cosmetic Ingredients
Frankfurt, Germany

[1] Present affiliation: DABMEB Consultancy, Ltd., Sharnbrook, U.K.
[2] Present affiliation: The University of Manchester, Manchester, U.K.

Preface

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers following repeated exposure to skin sensitizing chemicals and products. ACD results in lost workdays[3] and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). To minimize the occurrence of ACD, regulatory authorities require testing to identify substances that may cause skin sensitization (**Appendix G**). Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary for workers and consumers to avoid development of ACD.

Skin sensitization testing has typically required the use of guinea pigs (Buehler 1965; Magnusson and Kligman 1970). However, in 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated an alternative test method known as the murine (mouse) local lymph node assay (LLNA). Based on the validation database and performance, ICCVAM recommended the LLNA as an alternative test method for assessing the skin sensitization potential of most types of substances (ICCVAM 1999). United States and international regulatory agencies subsequently accepted the LLNA as a valid alternative test method for ACD testing. The LLNA provides several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time to perform, and availability of dose-response information. The LLNA is now used around the world.

In 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to evaluate several new versions and applications of the LLNA, including use of the LLNA for determining skin sensitization potency categories. CPSC based the nomination on their interest in assessing the usefulness and limitations of the LLNA for identifying chemicals and products likely to be strong human skin sensitizers. ICCVAM assigned the nomination a high priority after considering favorable comments from the public and ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). As part of the NICEATM-ICCVAM collaboration with the European Centre for Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM), scientists from these centers served as liaisons on the ICCVAM interagency Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA potency evaluation is included with this report (**Appendix A**).

This test method evaluation report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA for potency categorization of chemicals causing ACD in humans. The database of substances used to evaluate the accuracy of the LLNA for correctly determining skin sensitization potency categories is discussed and summarized.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the evaluation process. ICCVAM considered the SACATM comments, the report of an independent international scientific peer review panel (Panel), and all public comments before finalizing the ICCVAM test method recommendations for use of the LLNA for determining skin sensitization potency categories. The recommendations and the background review document, which is provided here as **Appendix C**, are incorporated in this ICCVAM test method evaluation report. As required by the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285*l*-3), ICCVAM will forward this report and its recommendations to U.S. Federal agencies for consideration and acceptance decisions, where appropriate. Federal agencies must respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. ICCVAM recommendations

[3] http://www.bls.gov/IIF

are available to the public on the NICEATM-ICCVAM website,[4] and agency responses will also be made available on the website as they are received.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for serving as the Panel Chair and to Dr. Michael Olson, Dr. Michael Woolhiser, and Kim Headrick for their service as Evaluation Group Chairs during the March 4-6, 2008, Panel meeting. We thank the interagency IWG for assuring a meaningful and comprehensive review. We especially thank Dr. Joanna Matheson (CPSC) and Dr. Abigail Jacobs (U.S. Food and Drug Administration Center for Drug Evaluation and Research) for serving as co-chairs of the interagency IWG. We also acknowledge Integrated Laboratory Systems, Inc., the NICEATM support contractor, for providing excellent scientific and operational support, including Dr. David Allen, Thomas Burns, Michael Paris, Dr. Eleni Salicru, Frank Stack, and Dr. Judy Strickland. Finally, we thank Drs. Silvia Casati and Alexandre Angers, and Dr. Hajime Kojima, the interagency IWG liaisons from ECVAM and JaCVAM, respectively, for their participation and contributions.

This ICCVAM evaluation of the LLNA for determining potency categories of skin-sensitizing chemicals is expected to assist regulatory agencies in determining when it may or may not be appropriate to use LLNA results for potency categorization and to facilitate regulatory agency decisions on the acceptability of the LLNA for this purpose. Appropriate use of the LLNA by industry is expected to significantly reduce and refine animal use required for ACD testing, while continuing to support the protection of human health.

Jodie A. Kulpa-Eddy, D.V.M.
APHIS-VS-NCIE-Agricultural Select Agent Program
U.S. Department of Agriculture
Acting Chair, ICCVAM

Rear Admiral William S. Stokes, D.V.M., DACLAM
Assistant Surgeon General, U.S. Public Health Service
Director, NICEATM
Executive Director, ICCVAM

[4] http://iccvam.niehs.nih.gov

Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the use of the murine (mouse) local lymph node assay (LLNA) as a stand-alone test method to determine skin sensitization potency categories. The LLNA is used to identify chemicals and products that may cause allergic contact dermatitis (ACD), an allergic skin reaction characterized by redness, swelling, and itching. This test method evaluation report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA for potency categorization of chemicals causing ACD in humans as well as recommendations for future studies. Also included in this report are a detailed timeline of the LLNA potency evaluation (**Appendix A**) and the final background review document (BRD) describing the validation status of the LLNA for this proposed usage (**Appendix C**).

Following a nomination by the U.S. Consumer Product Safety Commission (CPSC) to assess the validation status of the LLNA as a stand-alone test method for potency determinations for classification purposes, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the ICCVAM interagency Immunotoxicity Working Group (IWG) prepared a draft BRD, and ICCVAM prepared draft test method recommendations. The CPSC, under the Federal Hazardous Substances Act, currently requires hazard labeling of only products that are considered to be strong skin sensitizers, based on a weight-of-evidence approach that considers frequency of responses in exposed human populations, severity of responses, and the dose at which allergic reactions occur (15 U.S.C. 1261). Criteria for test results from animal studies that could be used to identify potential strong human skin sensitizers would be helpful for the purposes of hazard identification for CPSC and other agencies with an interest in identifying strong skin sensitizers. Accordingly, ICCVAM evaluated the extent that LLNA results could be used to correctly predict strong versus other than strong human skin sensitizers as detailed in the BRD.

The draft BRD and draft ICCVAM test method recommendations were provided to an independent international scientific peer review panel (Panel) and the public for their consideration. The Panel met in public session on March 4-6, 2008, to discuss its review of the draft BRD and to provide conclusions and recommendations regarding the validation status of the LLNA as a stand-alone test method to determine skin sensitization potency categories. The Panel also reviewed how well the information in the draft BRD supported ICCVAM's draft test method recommendations. The Panel agreed with ICCVAM that the LLNA should not be used as a stand-alone test method for categorizing skin sensitizers based on potency but that it can be used as part of a weight-of-evidence evaluation for this purpose. The Panel recommended that NICEATM perform additional analyses using alternative human reference values that might be more appropriate for evaluating the use of the LLNA for skin sensitization potency determinations.

NICEATM performed these analyses for the final BRD, which is included as **Appendix C**, and ICCVAM finalized the test method recommendations. In finalizing this test method evaluation report and the BRD, ICCVAM considered (1) the conclusions and recommendations of the Panel, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and (3) public comments.

ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the LLNA can be used to categorize substances as strong sensitizers (Globally Harmonized System of Classification and Labelling of Chemicals [GHS] Subcategory 1A) when the estimated concentration that produces a positive LLNA result (i.e., EC3) is ≤2%. However, because almost half (48% [13/27]) of the known strong human skin sensitizers have an EC3 > 2% or are negative in the LLNA, the LLNA cannot be considered a stand-alone assay to categorize skin

sensitization potency. Additional information is required to categorize a substance as other than a strong sensitizer (GHS Subcategory 1B: "other" skin sensitizers) when the substance produces an LLNA EC3 > 2%. These recommendations are based on an accuracy analysis (see **Section 3.4**) that included 136 substances for which there were both LLNA and human data (i.e., 27 strong human skin sensitizers, 49 other than strong human skin sensitizers, and 60 human nonsensitizers).

ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends use of the recently updated LLNA test method protocol (**Appendix B**), which includes improved dose selection procedures to guide selection of the highest dose that will aid in minimizing false negatives, and the procedures for calculating the EC3. The updated LLNA test method protocol provides for a 20% reduction in the number of animals required compared to the previously recommended LLNA protocol by reducing the number of required animals per group from five to four. Further, the collection of individual animal data and inclusion of both a concurrent vehicle and positive control are recommended for each study.

ICCVAM Recommendations: Future Studies

To further evaluate the usefulness and limitations of the LLNA for potency determinations, efforts should be made to identify additional high-quality human test data and experience for substances with comparative LLNA data. Emphasis should be placed on identifying substances that are classified as strong skin sensitizers based on a human threshold induction concentration of <500 μg/cm^2 to more adequately evaluate the LLNA EC3 value that will best distinguish strong from other than strong skin sensitizers. ICCVAM encourages the development, validation, and evaluation of integrated decision strategies that consider other types of relevant information such as quantitative structure-activity relationships, structural alerts, peptide reactivity, *in vitro* testing data, human data or experience, and related existing data from similar chemical entities.

Validation Status of the Use of the LLNA to Determine Skin Sensitization Potency Categories

The extent to which the LLNA correctly classifies strong versus other than strong human skin sensitizers was evaluated using a database of 136 substances with both LLNA and human data. The dose per skin area, which represents a defined incidence of a positive response among test subjects (i.e., 5%, DSA_{05} value) from the human maximization test or human repeat-insult patch test, was used as the human threshold response because it was viewed as analogous to the EC3 value, which is also a threshold positive response.

The 76 human sensitizers (among the 136 substances with LLNA and human data) were categorized as either "strong" or "other" sensitizers using the GHS criteria: $DSA_{05} \leq 500$ μg/cm^2 for strong sensitizers (GHS Subcategory 1A) and $DSA_{05} > 500$ μg/cm^2 for other sensitizers (GHS Subcategory 1B) (UN 2009). Of the 27 strong human sensitizers, 14 had LLNA EC3 ≤ 2%, 11 had EC3 > 2%, and two were negative in the LLNA. Forty-nine human sensitizers were other sensitizers: three with LLNA EC3 ≤ 2%, 35 with EC3 > 2%, and 11 with negative LLNA results. Of the 60 human nonsensitizers, 35 were sensitizers in the LLNA (four with LLNA EC3 ≤ 2%, 31 with EC3 > 2%), and 25 were nonsensitizers in the LLNA.

The correct classification, underclassification, and overclassification rates[5] of the LLNA versus human data were initially calculated using the GHS criteria of EC3 ≤ 2% for strong sensitizers and EC3 > 2% for other sensitizers. Based on this database, the LLNA correctly identified 52% (14/27) of

[5] The correct classification rate is the proportion of substances that are correctly assigned to a human potency category by the LLNA result. The underclassification rate is the proportion of substances that are incorrectly assigned to a less severe human potency category by the LLNA result, and the overclassification rate is the proportion of substances that are incorrectly assigned to a more severe human potency category by the LLNA result.

the strong human sensitizers using EC3 ≤ 2%, but underclassified 48% (13/27) (see **Appendix C, Section 6.1.2**). Among the 21 substances that produced an EC3 ≤ 2%, 67% (14/21) were strong human skin sensitizers (GHS Subcategory 1A), but the remaining 33% (7/21) were either other human skin sensitizers (GHS Subcategory 1B, n = 3) or substances not classified as human skin sensitizers (n = 4).

Of the 13 strong human sensitizers that were not categorized as strong sensitizers using the GHS criterion of LLNA EC3 ≤ 2%, 77% (10/13) produced an LLNA EC3 value between 2% and 10%, one produced an LLNA EC3 of 30.9%, and two were negative in the LLNA. The 13 substances shared the following commonalities with regard to physicochemical characteristics:

- Twelve of 13 had molecular weights within a range of 100 (12/13 substances had molecular weights of 98.15 to 192.3).
- Eight of the 13 substances were liquids.
- All six of the substances for which peptide reactivity information was available had high (n = 5) or moderate (n = 1) peptide reactivity.

As noted above, most (77%) of the strong human sensitizers that were underclassified by the LLNA (10/13) had EC3 values between 2% and 10%. Use of LLNA EC3 ≤ 10% to classify substances as strong sensitizers correctly classified 89% (24/27) of the strong sensitizers compared with the 52% (14/27) of the strong sensitizers correctly classified using EC3 ≤ 2%. However, it also decreased the number of other than strong sensitizers classified correctly (31% [15/49] versus 71% [35/49]). The optimum EC3 value (3.8%) resulted in the highest correct classification rate for strong human sensitizers, other than strong human sensitizers, and nonsensitizers combined (55% [75/136]). The lowest underclassification rate was for strong and other than strong skin sensitizers (22% [17/76]).

ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process for use of the LLNA as a stand-alone test method to determine skin sensitization potency categories included a public review meeting by an independent scientific peer review panel, multiple opportunities for public comments, and comments from SACATM. ICCVAM and the interagency IWG considered the Panel report, the SACATM comments, and all public comments before finalizing the ICCVAM test method evaluation report and BRD.

This page intentionally left blank

1.0 Introduction

1.1 Background Information on the Murine Local Lymph Node Assay

The murine (mouse) local lymph node assay (LLNA) is an alternative skin sensitization test method that requires fewer animals and less time than the traditionally accepted guinea pig tests, the guinea pig maximization test and the Buehler test (EPA 2003; OECD 1992). It also avoids animal discomfort that can occur in the guinea pig tests when substances cause allergic contact dermatitis (ACD). The LLNA measures cell proliferation in the draining auricular lymph nodes of the mouse by analyzing incorporation of a radioactive marker into newly synthesized DNA. The LLNA was the first alternative test method evaluated and recommended by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). International regulatory authorities have now recognized the LLNA as an acceptable alternative to guinea pig tests for most testing situations.

The use of the LLNA as a stand-alone test method to determine skin sensitization potency categories is one of several LLNA-related topics nominated by the U.S. Consumer Product Safety Commission (CPSC) for evaluation by ICCVAM together with the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).[6] This evaluation assessed the accuracy of the LLNA to correctly determine skin sensitization potency in humans.

1.2 ICCVAM, NICEATM, and Interagency Immunotoxicity Working Group

In accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 U.S.C. 285*l*-3), ICCVAM coordinates the technical evaluations of new, revised, and alternative test methods with regulatory applicability. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM's activities. After considering comments from the public and ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), ICCVAM members unanimously agreed that an evaluation of the LLNA as a stand-alone test method to determine skin sensitization potency categories should have a high priority. A detailed timeline of this evaluation is provided in **Appendix A**.

ICCVAM established an interagency Immunotoxicity Working Group (IWG) to work with NICEATM to evaluate the use of the LLNA as a stand-alone test method to determine skin sensitization potency categories. The European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) designated liaison members to the interagency IWG.

A May 17, 2007, *Federal Register* (FR) notice (72 FR 27815)[7] requested data and information on these test methods and nominations of individuals to serve on an independent international scientific peer review panel (Panel). The request was also disseminated via the ICCVAM electronic mailing list and through direct requests to over 100 stakeholder organizations. In response to this request, a Panel of 19 experts representing eight countries was formed. The expertise of the Panel included alternative toxicity test methods, animal welfare, biostatistics, dermal toxicity, dermatology, human health risk assessment, immunotoxicology, pharmacology, regulatory toxicology, and occupational and environmental health.

1.3 LLNA Background Review Document

To facilitate peer review of the evaluation of the LLNA, the interagency IWG and NICEATM prepared a comprehensive draft background review document (BRD) that provided information and data from validation studies and the scientific literature. The final BRD is provided in **Appendix C**.

[6] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[7] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

The draft BRD examined data derived from a database of over 500 substances tested in the LLNA. For each substance with comparative human reference data, skin sensitization potency was evaluated by comparing the LLNA EC3 value, the estimated concentration of a substance expected to produce a stimulation index (SI) of 3, the threshold value for a substance to be considered a sensitizer in the LLNA (Kimber et al. 2001), to the threshold concentration inducing a human response. On January 8, 2008, ICCVAM announced the availability of the draft BRD to the public.

1.4 Peer Review Panel

ICCVAM announced a March 4-6, 2008, public peer review panel (Panel) meeting to review the validation status of the LLNA as a stand-alone test method to determine skin sensitization potency categories (and other LLNA-related activities) (73 FR 1360).[8] All of the information provided to the Panel, including the draft BRD, ICCVAM draft test method recommendations, and all public comments received before the Panel meeting, were made publicly available via the NICEATM-ICCVAM website.[9]

The Panel evaluated (1) the extent to which the draft BRD addressed established validation and acceptance criteria and (2) the extent to which the draft BRD supported ICCVAM's draft proposed test method uses, recommended test method protocol, and proposed future studies. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. The Panel considered these comments as well as those submitted prior to the meeting before concluding their deliberations. As indicated in the Panel report (**Appendix D**), the Panel agreed with the ICCVAM draft recommendations that the LLNA should not be used as a stand-alone assay for categorizing skin sensitizers based on potency but that it could be used as part of a weight-of-evidence evaluation for this purpose. The Panel further recommended that NICEATM perform additional analyses using alternative human reference values that might be more appropriate for evaluating the use of the LLNA for skin sensitization potency determinations. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations[10] (**Appendix D**) on the NICEATM-ICCVAM website for public review and comment (announced in 73 FR 29136).[11]

1.5 Scientific Advisory Committee on Alternative Toxicological Methods

The Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) is a Federally chartered advisory committee that advises ICCVAM, NICEATM, and the Director of the NIEHS.[12] SACATM provides advice on priorities and activities related to the development, validation, scientific review, regulatory acceptance, implementation, and national and international harmonization of new, revised, and alternative toxicological test methods. The NIEHS Director appoints voting members to SACATM, which includes representatives from academia, state government, industry, and animal protection organizations.

ICCVAM provided SACATM with the draft BRD and draft test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 18-19, 2008, where public stakeholders were given another opportunity to comment.

[8] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[9] http://iccvam.niehs.nih.gov
[10] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[11] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf
[12] http://ntp.niehs.nih.gov/index.cfm?objectid=720165EC-BDB7-CEBA-F517D1DEE4D7D129

1.6 Final ICCVAM Test Method Recommendations and Final Background Review Document

ICCVAM and the interagency IWG considered the SACATM comments, the Panel report, and all public comments before finalizing ICCVAM test method recommendations for use of the LLNA as a stand-alone test method to determine skin sensitization potency categories. The recommendations (**Section 2.0**) and the final BRD (**Appendix C**) are incorporated in this ICCVAM test method evaluation report. As required by the ICCVAM Authorization Act of 2000, ICCVAM will forward this report and its recommendations to U.S. Federal agencies for consideration. Within 180 days after receiving ICCVAM test method recommendations, Federal agencies must respond to ICCVAM regarding their consideration and acceptance decisions, where appropriate. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website,[13] and agency responses will be made available as they are received.

[13] http://iccvam.niehs.nih.gov

2.0 ICCVAM Recommendations: Usefulness and Limitations of the LLNA for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans, Test Method Protocol, and Future Studies

ICCVAM has completed its evaluation of the validation status of the LLNA to classify substances into skin sensitization potency categories. NICEATM and ICCVAM prepared a comprehensive BRD that includes the data and information available to characterize the validity of this proposed use of the LLNA. The information included in the BRD (**Appendix C**) is based on a review of 136 substances with LLNA data and either (1) human maximization test (HMT) data (Kligman 1966; Kligman and Epstein 1975), (2) human repeat-insult patch test (HRIPT) data (Marzulli and Maibach 1974; Politano and Api 2008), or (3) other human data (for nonsensitizer status only). The database represents 76 human skin sensitizers and 60 human nonsensitizers, with 63 substances classified as skin sensitizers by both LLNA and human data.

The third revised edition of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) classifies skin sensitizers as Category 1 (UN 2009). Category 1 can be further subcategorized into 1A ("strong" skin sensitizers) and 1B ("other" skin sensitizers) based on results from human studies and/or animal studies (i.e., the LLNA and guinea pig tests). Under the GHS classification system, substances with positive responses in the HMT or HRIPT at induction thresholds $\leq$500 $\mu g/cm^2$ are classified as Subcategory 1A, and substances with positive responses at induction thresholds >500 $\mu g/cm^2$ are classified as Subcategory 1B. The GHS also provides criteria for assigning these categories based on positive results in the LLNA using the EC3 value (i.e., the estimated concentration of a substance expected to produce an SI of 3, the threshold value for a substance to be considered a sensitizer in the LLNA) as the metric for relative potency (Kimber et al. 2001). Substances that produce an EC3 $\leq$ 2% are classified as Subcategory 1A, and substances with an EC3 > 2% are classified as Subcategory 1B (UN 2009). Nonsensitizers are not classified.

Most authorities do not currently regulate products based on skin sensitization potency, instead classifying them simply as "yes" or "no" for skin sensitization hazard. Under the Federal Hazardous Substances Act (15 U.S.C. 1261), CPSC currently requires hazard labeling of only those products considered to be strong skin sensitizers based on a weight-of-evidence approach that considers frequency of responses in exposed human populations, severity of responses, and the dose at which allergic reactions occur.[14] Criteria for test results from animal studies that could be used to identify potential strong human skin sensitizers would help in hazard identification for CPSC and other agencies with an interest in identifying strong skin sensitizers. Accordingly, ICCVAM evaluated the extent to which LLNA results could correctly predict strong versus other than strong human skin sensitizers.

2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the LLNA, using the GHS classification criteria, can be used to categorize substances as strong sensitizers (GHS Subcategory 1A) when the estimated concentration that produces a positive LLNA result (i.e., EC3) is $\leq$2%. However, because almost half of the known strong human skin sensitizers have an EC3 > 2%, the LLNA cannot be considered a stand-alone assay to determine skin sensitization potency categories. Additional information is required to categorize a

[14] Substances that meet the CPSC's definition of strong sensitizer: (1) 4-phenylenediamine and products containing it; (2) powdered orris root and products containing it; (3) epoxy resins systems containing, in any concentration, ethylenediamine, diethylenetriamine, and diglycidyl ethers with molecular weight less than 200; (4) formaldehyde and products containing ≥1%; and (5) oil of bergamot and products containing ≥2% (16 C.F.R. 1500.13).

substance as other than a strong sensitizer (GHS Subcategory 1B: "other" skin sensitizer) when the substance produces an LLNA EC3 > 2%.

These recommendations are based on an accuracy analysis (see **Section 3.4**) that included 136 substances for which there were both LLNA and human data (i.e., 27 strong human skin sensitizers, 49 other than strong human skin sensitizers, and 60 human nonsensitizers). Using the GHS criteria of LLNA EC3 ≤ 2% to classify substances as strong sensitizers and EC3 > 2% to classify substances as other than strong sensitizers, the overall correct prediction of human potency categories (i.e., strong sensitizers, other than strong sensitizers, and nonsensitizers) was 54% (74/136).

The LLNA EC3 ≤ 2% correctly identified 52% (14/27) of the strong human skin sensitizers. However, 48% (13/27) of strong human skin sensitizers were underclassified by the LLNA as either other than strong skin sensitizers (i.e., LLNA EC3 > 2%) or as nonsensitizers (i.e., negative in the LLNA). Among the 21 substances that produced an LLNA EC3 ≤ 2%, 67% (14/21) were correctly identified as strong sensitizers, but 33% (7/21) were incorrectly overclassified as strong skin sensitizers based on available human test data. Four of the seven substances were not classified as skin sensitizers (nonsensitizers) based on human test data.

Most substances with EC3 values between 2% and 10% should be considered to have the potential to be strong skin sensitizers unless there are data to support categorization as other than strong skin sensitizers. Of the strong human skin sensitizers in this database, 37% (10/27) produced EC3 values between 2% and 10%, which accounts for 76% (10/13) of the strong sensitizers that were underclassified by the LLNA. Therefore, it is likely that a considerable number of strong human skin sensitizers within the broader population of chemicals may produce EC3 values within this range.

By comparison, when the LLNA EC3 criterion for identifying strong skin sensitizers was increased to EC3 ≤ 10%, 89% (24/27) of the strong human skin sensitizers were correctly classified by the LLNA, and only 11% (3/27) were underclassified.

2.2 ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends use of the recently updated LLNA test method protocol (**Appendix B**), which includes improved dose selection procedures to guide selection of the highest dose that will help minimize false negatives. The updated LLNA test method protocol provides for a 20% reduction in the required number of animals compared to the previously recommended LLNA protocol (ICCVAM 2001). The updated protocol reduces the number of required animals per group from five to four. It also recommends collection of individual animal data and inclusion of both a concurrent vehicle and a positive control in each study. These protocol modifications have resulted in an overall reduction of 20% in the number of animals used in a given test.

2.3 ICCVAM Recommendations: Future Studies

To further evaluate the usefulness and limitations of the LLNA for skin sensitization potency categorization, efforts should be made to identify additional high-quality human test data and human experience for substances with LLNA data for comparison. Emphasis should be placed on identifying substances that are classified as strong skin sensitizers based on a human threshold induction concentration of <500 $\mu g/cm^2$ to better evaluate the LLNA EC3 value that will best distinguish strong from other than strong human skin sensitizers. In order to develop a more accurate assessment of strong human skin sensitizers using LLNA results, especially for substances that produce an EC3 value between 2% and 10%, ICCVAM encourages the development, validation, and evaluation of integrated decision strategies that consider other types of relevant information such as quantitative structure-activity relationships, structural alerts, peptide reactivity, *in vitro* testing data, human test data or experience, and existing data from similar chemical entities.

3.0 Validation Status for Use of the LLNA to Determine Skin Sensitization Potency Categories

The ICCVAM BRD (**Appendix C**) provides a comprehensive review of the validation status of the LLNA to determine skin sensitization potency categories. The BRD details the substances analyzed in the validation database, the accuracy and reliability of the LLNA for potency categorization, and all available data supporting its validity for the purpose of determining skin sensitization potency categories. This section summarizes the evaluation and validation status detailed in the BRD.

3.1 Test Method Description

The LLNA test method identifies potential skin sensitizers by quantifying lymphocyte proliferation in the draining auricular lymph nodes during the induction phase of skin sensitization. The magnitude of lymphocyte proliferation then correlates with the extent to which sensitization develops after topical exposure to the potential skin sensitizer. For the purposes of this analysis, relative potency in the LLNA is defined as the concentration of a fixed volume of a substance that is required for the induction phase of a skin sensitization reaction to occur. The more potent the substance the smaller the concentration needed.

3.1.1 General Test Method Procedures

The recently updated ICCVAM-recommended test method protocol for the LLNA describes the conduct of the assay in detail (**Appendix B**). A test substance-induced increase in lymphocyte proliferation in the draining lymph nodes of the ear, the site of application, is used to identify chemical sensitizers. Mice are injected with radiolabeled thymidine (or an analogue of thymidine), which is incorporated into the DNA of proliferating cells. The SI, the ratio of incorporated radioactivity in the auricular lymph nodes of treated versus control mice, is used to assess the sensitizing potential of the test substance. An SI ≥ 3 is used to classify a test substance as a skin-sensitizing agent. In the LLNA, a volume of 25 μL of the test substance is applied to each ear, and the estimated concentration expected to produce an SI of 3 (i.e., the EC3) is used as the metric for predicting skin-sensitization potency. Most recently, variations of the LLNA that do not employ radioactivity have also been evaluated and recommended by ICCVAM (ICCVAM 2010b, 2010a) and adopted as OECD test guidelines (OECD 2010b, 2010c). However, these nonradioactive LLNA methods have not been evaluated for skin sensitization potency determinations.

3.2 Validation Database

The validation database used to evaluate the LLNA's capacity to determine skin sensitization potency categories consists of 196 substances that have LLNA data with comparative guinea pig data, human data, or both. Data were obtained from published reports and unpublished data submitted to NICEATM in response to a *Federal Register* notice (72 FR 27815).[15] These 196 substances include 136 substances with comparative human data (76 sensitizers, 60 nonsensitizers), 116 substances with comparative guinea pig data (64 sensitizers, 52 nonsensitizers), and 56 substances with comparative human and guinea pig data (35 human sensitizers, 21 human nonsensitizers). Among the 136 substances with comparative human data and the 56 substances with comparative human and guinea pig data are 4-phenylenediamine and formaldehyde, two of the five substances that meet CPSC's definition of strong sensitizer (16 C.F.R. 1500.13).

Table 3-1 shows the chemical classes represented by the 196 substances tested in the LLNA with human and/or guinea pig skin sensitization data. Considering inorganics as one class, the 196 substances represent 30 chemical classes. Fifty-five substances are classified in more than one

[15] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

chemical class. The classes with the highest number of substances are carboxylic acids (33 substances) and aldehydes (18 substances). In the entire NICEATM LLNA database of more than 600 substances (a sufficiently large representation for further analyses), 22 chemical classes are represented by at least five substances. Twenty of these classes have at least 60% of the LLNA results identified as positive (i.e., SI $\geq$ 3). These 20 classes are identified as those most likely to be associated with skin sensitization. In comparison, 19 of these 20 classes are also represented in the database of 196 substances included in this evaluation (i.e., the NICEATM LLNA potency database); only the class of macromolecular substances is not included. Further, all of the chemical classes previously found to contain common skin allergens (e.g., aldehydes, ketones, quinones, and acrylates) (Gerberick et al. 2004) are represented in this LLNA potency evaluation. **Annex III** of the BRD (**Appendix C**) provides the chemical classes to which each substance was assigned, information on the physicochemical properties (e.g., estimated log octanol-water partition coefficient), Chemical Abstracts Service Registry Number, and uses. Some substances were assigned to more than one chemical class, and some were not assigned to a specific chemical class.

Table 3-1 Chemical Classes Represented in the LLNA Potency Database

Chemical Class	No. of Substances[1]
Inorganic chemicals	**11**
Aluminum compounds	1
Chromium compounds	1
Elements	1
Gold compounds	1
Manganese compounds	1
Mercury compounds	1
Metals	5
Sulfur compounds	1
Zinc compounds	1
Organic chemicals	**185**
Alcohols	15
Aldehydes	18
Amides	5
Amines	16
Anhydrides	2
Azo compounds	5
Carbohydrates	6
Carboxylic acids	33
Cyanates	1
Esters	5
Ethers	6
Formulations[2]	16
Heterocyclic compounds	15
Hydrocarbons, acyclic	5
Hydrocarbons, cyclic	12
Hydrocarbons, halogenated	1
Hydrocarbons, other	9
Ketones	3
Lactones	1
Lipids	15
Natural complex substances[2]	15
Nitriles	2
Nitro compounds	2
Onium compounds	1
Phenols	14
Polycyclic compounds	4
Quinones	1
Sulfur compounds	16
Ureas	2
Unknown[3]	**3**

Abbreviations: LLNA = murine local lymph node assay; No. = number.

Chemical classifications are based on the Medical Subject Headings classification for chemicals and drugs developed by the National Library of Medicine (http://www.nlm.nih.gov/mesh/meshhome.html).

[1] The total number of substances assigned to each chemical class does not equal the total number of substances evaluated because some substances were assigned to more than one chemical class and some substances were not assigned to a specific chemical class.

[2] Substances assigned to these classes were mixtures of two or more components. In some cases, another chemical class was also assigned based on the active ingredient (for formulations) or the principal component (for natural complex substances).

[3] The proprietary substances (fatty acid glutamate, fatty acid alcohol #1, and fatty acid alcohol #2) were not identified sufficiently for a chemical class to be assigned.

3.3 Reference Test Method Data

The reference database for this evaluation consisted of (1) clinical studies that used the HMT or HRIPT or (2) other human information (for nonsensitizer status only). In the HMT and the HRIPT, potency information is determined from the no observed effect level (NOEL), the lowest observed effect level (LOEL), or the induction dose per skin area (DSA) that produces a positive response in 5% of the tested population (DSA_{05}). The third revised edition of the GHS classifies skin sensitizers as Category 1 (UN 2009) (see **Appendix E**). Category 1 substances are further subcategorized into 1A ("strong" skin sensitizers) or 1B ("other" skin sensitizers) based on results from human and/or animal studies (i.e., LLNA and guinea pig tests). Under the GHS classification system, substances with positive responses in the HMT or HRIPT at induction thresholds $\leq$500 $\mu g/cm^2$ are classified as Subcategory 1A, and substances with positive responses at >500 $\mu g/cm^2$ are classified as Subcategory 1B. The GHS criteria for using the LLNA to subcategorize sensitizers is based on the EC3 value: substances with EC3 $\leq$ 2% are classified as Subcategory 1A ("strong" skin sensitizers) and substances with EC3 > 2% are classified as Subcategory 1B ("other" skin sensitizers) (UN 2009). Nonsensitizers are not classified.

3.4 Test Method Accuracy

3.4.1 DSA_{05} and EC3 Values

The DSA_{05} value represents a defined incidence of a positive response among test subjects (i.e., 5%). It was used as the human threshold response because it corresponds best (compared with NOEL or LOEL values) to the EC3 value, which is also a threshold positive response. More than one LLNA test, often in different vehicles, was available for many of the substances in the validation database. Single EC3 and DSA_{05} values were established for each substance (see **Appendix C, Annex II-4**) before any analyses were conducted. Geometric mean EC3 and DSA_{05} values for each substance with multiple results were favored over the most potent EC3 and DSA_{05} values because the coefficient of determination, R^2, was higher for the geometric mean EC3 and DSA_{05} regression (0.448 versus 0.382; see **Appendix C, Section 6.1.1**). Geometric mean EC3 values were calculated regardless of vehicle because statistical analyses showed that vehicle had no impact on the relationship of LLNA EC3 and human DSA_{05} values for the substances tested (see **Appendix C, Annex IV**).

Forty-seven of the 98 substances with positive LLNA results had multiple EC3 values. The number of values for each substance ranged from 2 to 66. Individual EC3 values ranged from 0.0007% to 98.5%. Substances with a majority of negative LLNA test results were not assigned EC3 values. For example, nickel salts and streptomycin were each considered negative in the LLNA because most of the LLNA responses were negative (8/10 tests for nickel salts; 4/5 tests for streptomycin). Likewise, substances with multiple positive HMT or HRIPT responses were assigned geometric mean DSA_{05} values calculated from all the available DSA_{05} values (see **Appendix C, Annex II-4**). Thirty-two of the 76 substances with positive human results had multiple DSA_{05} values. The number of values ranged from 2 to 8. Individual DSA_{05} values ranged from 1.9 to 335545 $\mu g/cm^2$.

Table 3-2 shows the distribution of substances into the GHS potency categories using geometric mean LLNA EC3 values and geometric mean DSA_{05} values for substances with multiple results. The 76 human sensitizers include 27 strong sensitizers (14 with LLNA EC3 $\leq$ 2%, 11 with EC3 > 2%, and two with negative LLNA results) and 49 other than strong sensitizers (three with LLNA EC3 $\leq$ 2%, 35 with EC3 > 2%, and 11 with negative LLNA results). Of the 60 human nonsensitizers, 35 were LLNA sensitizers (four with LLNA EC3 $\leq$ 2%, 31 with EC3 > 2%) and 25 were LLNA nonsensitizers. **Figure 3-1** shows geometric mean LLNA EC3 values plotted against the geometric mean DSA_{05} values for the 63 LLNA and human sensitizers. Concordant LLNA and human nonsensitizers, LLNA false positives, and LLNA false negatives are shown on the edges of the graph.

The GHS cutoffs, EC3 ≤ 2% and DSA_{05} ≤ 500 μg/cm^2, are marked to show the correspondence of the data with the GHS classification criteria for Subcategories 1A and 1B.

Table 3-2 Distribution of 136 Substances for Classification Rate Analyses[1]

LLNA + /Human +		LLNA + / Human -	LLNA - / Human +	LLNA - / Human -
Strong[2]	**Other[3]**			
25 (14 EC3 ≤ 2%; 11 EC3 > 2%)	38 (3 EC3 ≤ 2%; 35 EC3 > 2%)	35 (4 EC3 ≤ 2%; 31 EC3 > 2%)	13 (2 strong; 11 other)[2,3]	25

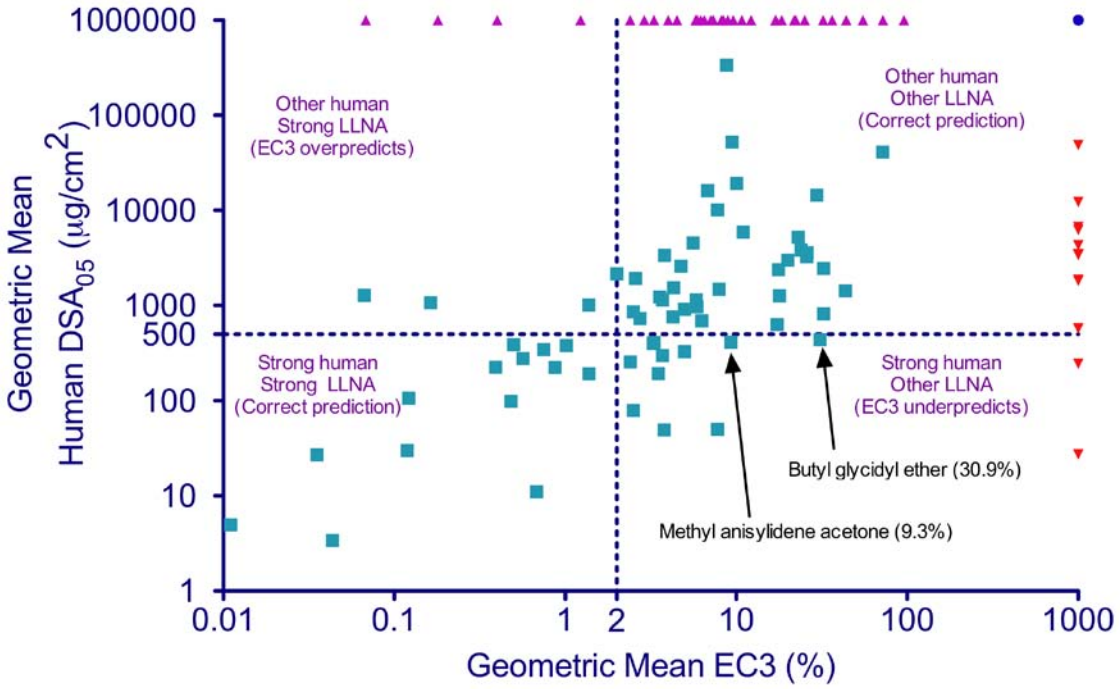
Abbreviations: DSA_{05} = induction dose per skin area, in μg/cm^2, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay.

[1] Classification based on geometric mean EC3 and DSA_{05} values.

[2] Human sensitizers were classified as strong sensitizers if DSA_{05} ≤ 500 μg/cm^2.

[3] Human sensitizers were classified as other sensitizers if DSA_{05} > 500 μg/cm^2.

Figure 3-1 LLNA EC3 and Human Results by GHS Potency Category for 136 Substances



Legend: ■ Human/LLNA sensitizers (n = 63); ▲ LLNA false positive (n = 35); ▼ LLNA false negative (n = 13); ● Concordant negative (n = 25).

Abbreviations: DSA_{05} = induction dose per skin area, in μg/cm^2, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, the threshold value for a substance to be considered a sensitizer in the LLNA; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2009); LLNA = murine local lymph node assay.

3.4.2 LLNA Classification of Strong and Other Than Strong Sensitizers in Humans

The extent to which the LLNA correctly classifies strong and other than strong sensitizers in humans was evaluated using the criteria for human thresholds defined in the GHS (UN 2009). The correct classification, underclassification, and overclassification rates of the LLNA versus human data were initially calculated using the GHS criteria of EC3 ≤ 2% for strong sensitizers and EC3 > 2% for other sensitizers. As indicated in **Tables 3-3** and **3-4**, based on this database, the LLNA correctly identified 52% (14/27) of the strong human skin sensitizers using EC3 ≤ 2% but underclassified 48% (13/27). Among the 21 substances that produced an EC3 ≤ 2%, 67% (14/21) were strong human skin sensitizers (GHS Subcategory 1A), but the remaining 33% (7/21) were either other than strong human skin sensitizers (GHS Subcategory 1B: n = 3) or substances not classified as human skin sensitizers (n = 4).

As indicated in **Figure 3-1**, most of the strong human skin sensitizers that were underclassified by the LLNA (10/13) had EC3 values from 2% to 10%. Therefore, the classification rates for human skin sensitizer categories obtained using incremental EC3 cutoff values up to 10% were also evaluated (**Table 3-3**). From EC3 ≤ 2% to ≤4%, the increase in number of correctly classified strong sensitizers (14 to 21) was almost directly proportional to the decrease in the number of correctly classified other than strong sensitizers (35 to 29). The number of human nonsensitizers overclassified as strong sensitizers increased from four to seven when the LLNA EC3 cutoff value moved from ≤2% to ≤4%. With each additional increase of 2% in the LLNA EC3 cutoff value, the number of correctly classified strong sensitizers increased by one substance. Using LLNA EC3 ≤ 10% to classify substances as strong sensitizers correctly classified 89% (24/27) of the strong sensitizers compared to the 52% (14/27) of the strong sensitizers correctly classified using EC3 ≤ 2% (**Table 3-4**). However, the proportion of substances classified by the LLNA as strong sensitizers that actually are strong human skin sensitizers was higher for EC3 ≤ 2% than for EC3 ≤ 10%: 67% (14/21) versus 36% (24/67) (see **Table 3-3**).

Figure 3-2 shows the change in the correct classification and underclassification rates for the 27 strong human skin sensitizers over the entire range of LLNA EC3 cutoff values. The correct potency classification rate for strong human skin sensitizers increased, and the underclassification rate decreased as the EC3 value increased. The correct classification rate plateaued, however, because the two strong human skin sensitizers that yielded negative results in the LLNA were not correctly classified by any EC3 cutoff value.

Of the 13 strong human skin sensitizers that were underclassified by the GHS criterion of LLNA EC3 ≤ 2%, 11 were underclassified as other sensitizers and two were underclassified as nonsensitizers. The two strong human skin sensitizers that were classified by the LLNA as nonsensitizers also yielded sensitizer results in a few LLNA tests (2/10 for nickel salts and 1/5 for streptomycin). However, the GHS criterion of EC3 ≤ 2% would have underclassified these strong human sensitizers even if their positive results had been used in the analysis. The two positive nickel results were for nickel sulfate in dimethyl sulfoxide (EC3 = 4.8%) and nickel chloride in 30% ethanol (EC3 = 5.5%). The positive result for streptomycin yielded EC3 = 33% in dimethylformamide. Ten of the 11 remaining discordant substances had EC3 values less than 10%. The substance with EC3 > 10% was butyl glycidyl ether (EC3 = 30.9%). The physicochemical commonalities among these 13 strong human skin sensitizers include molecular weights within a range of 100 (12/13 substances had molecular weights of 98.15 to 192.3). Eight of the 13 substances were liquids; and all six of the substances for which peptide reactivity information was available had high (n = 5) or moderate (n = 1) peptide reactivity.

Table 3-3 Concordance of LLNA and Human Data for Strong Sensitizer, Other Sensitizer, and Nonsensitizer Categories for 136 Substances at Selected LLNA EC3 Values

		Strong Sensitizer	Other Sensitizer	Nonsensitizer	Total
		EC3 ≤ 2% (GHS)	**EC3 > 2% (GHS)**	**Negative LLNA**	
Human Data[1]	Strong Sensitizer	14	11	2	27
	Other Sensitizer	3	35	11	49
	Nonsensitizer	4	31	25	60
	Total	21	77	38	136
		EC3 ≤ 4%	**EC3 > 4%**	**Negative LLNA**	
Human Data[1]	Strong Sensitizer	21	4	2	27
	Other Sensitizer	9	29	11	49
	Nonsensitizer	7	28	25	60
	Total	37	61	38	136
		EC3 ≤ 6%	**EC3 > 6%**	**Negative LLNA**	
Human Data[1]	Strong Sensitizer	22	3	2	27
	Other Sensitizer	16	22	11	49
	Nonsensitizer	12	23	25	60
	Total	50	48	38	136
		EC3 ≤ 8%	**EC3 > 8%**	**Negative LLNA**	
Human Data[1]	Strong Sensitizer	23	2	2	27
	Other Sensitizer	20	18	11	49
	Nonsensitizer	16	19	25	60
	Total	59	39	38	136
		EC3 ≤ 10%	**EC3 > 10%**	**Negative LLNA**	
Human Data[1]	Strong Sensitizer	24	1	2	27
	Other Sensitizer	23	15	11	49
	Nonsensitizer	20	15	25	60
	Total	67	31	38	136

Abbreviations: EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, the threshold value for a substance to be considered a sensitizer in the LLNA; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2009); LLNA = murine local lymph node assay.

[1] Human sensitizer data were DSA_{05} values (induction dose per skin area, in μg/cm^2, that produced a positive response in 5% of the tested population in a human repeat-insult patch test or human maximization test). Sensitizers were classified as strong if $DSA_{05} \leq 500$ μg/cm^2 and other if $DSA_{05} > 500$ μg/cm^2.

Thirteen substances that had LLNA EC3 > 2% or were nonsensitizers in the LLNA were strong human skin sensitizers. Fourteen percent (11/77) of the substances with EC3 > 2% were strong human skin sensitizers ($DSA_{05} \leq 500$ μg/cm^2). Five percent (2/38) of the substances that were negative in the LLNA were strong human skin sensitizers.

To determine the optimum EC3 value that could be used to identify strong and other than strong sensitizers, receiver-operator characteristic calculations (Fawcett 2006) were performed. The optimum EC3 value was defined as the value that resulted in the highest correct classification rate for strong human skin sensitizers, other human skin sensitizers, and nonsensitizers combined. The highest correct classification rate, 55% (75/136), occurred at both EC3 ≤ 3.8% and EC3 ≤ 3.5%. EC3 ≤ 3.8% was considered the optimum value based on the fact that it produced a lower underclassification rate for strong and other than strong skin sensitizers than EC3 ≤ 3.5%: 22% (17/76) versus 25% (19/76). These analyses are detailed in **Appendix C, Section 6.1.2**.

Table 3-4 Classification Rates for the Prediction of Human Potency Categories by Selected LLNA EC3 Cutoff Values[1] for 136 Substances

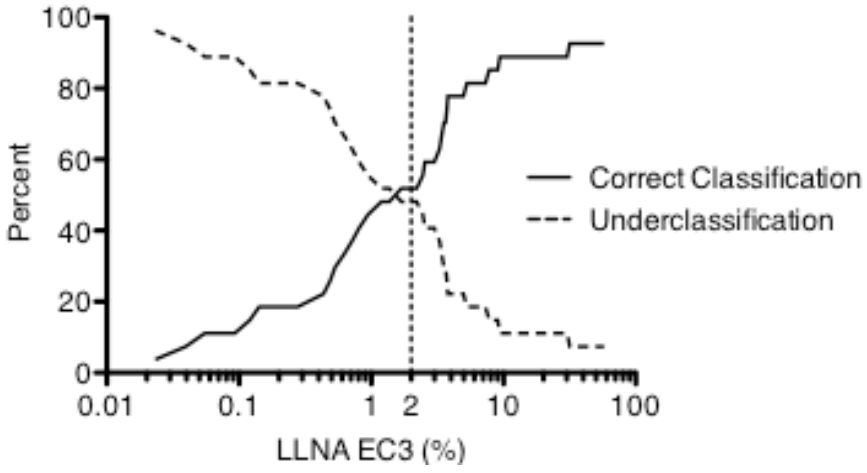
EC3 Cutoff for Strong versus Other Sensitizers	Strong Human Sensitizers ($DSA_{05} \leq 500$ µg/cm^2)		Other Human Sensitizers ($DSA_{05} > 500$ µg/cm^2)			Human Nonsensitizers		Overall Correct Potency Classification[2]
	Correct	Under	Over	Correct	Under	Correct	Over	
GHS Cutoff EC3 ≤ 2%	52 ± 19% (14/27)	48 ± 19% (13/27)	6 ± 7% (3/49)	71 ± 13% (35/49)	22 ± 12% (11/49)	42 ± 12% (25/60)	58 ± 12% (35/60)	54 ± 8% (74/136)
EC3 ≤ 4%	78 ± 16% (21/27)	22 ± 16% (6/27)	18 ± 11% (9/49)	59 ± 14% (29/49)	22 ± 12% (11/49)	42 ± 12% (25/60)	58 ± 12% (35/60)	54 ± 8% (74/136)
EC3 ≤ 6%	81 ± 15% (22/27)	19 ± 15% (5/27)	33 ± 13% (16/49)	45 ± 14% (22/49)	22 ± 12% (11/49)	42 ± 12% (25/60)	58 ± 12% (35/60)	50 ± 8% (68/136)
EC3 ≤ 8%	85 ± 13% (23/27)	15 ± 13% (4/27)	41 ± 14% (20/49)	37 ± 13% (18/49)	22 ± 12% (11/49)	42 ± 12% (25/60)	58 ± 12% (35/60)	48 ± 8% (65/136)
EC3 ≤ 10%	89 ± 12% (24/27)	11 ± 12% (3/27)	47 ± 14% (23/49)	31 ± 13% (15/49)	21 ± 12% (11/49)	42 ± 12% (25/60)	58 ± 12% (35/60)	47 ± 8% (64/136)

Abbreviations: DSA_{05} = induction dose per skin area, in µg/cm^2, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, the threshold value for a substance to be considered a sensitizer in the LLNA; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2009); LLNA = murine local lymph node assay.

[1] Classification rates are shown ±95% confidence limits.

[2] The overall correct classification rate includes the correct classification of strong human sensitizers, other than strong sensitizers, and nonsensitizers.

Figure 3-2 Classification Rates for the LLNA EC3 Prediction of 27 Strong Human Sensitizers



Abbreviations: EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay.

Analysis was based on 27 substances identified as strong sensitizers in humans using the human maximization test and/or the human repeat-insult patch test because the induction dose per skin area that produced a positive response in 5% of the tested population was ≤500 µg/cm^2.

Fifty-six substances had LLNA, guinea pig (i.e., the guinea pig maximization test and/or the Buehler test), and human skin sensitization data. The overall correct classification rate of the LLNA, using EC3 ≤ 2% to classify substances as strong skin sensitizers and EC3 > 2% to classify substances as other than strong skin sensitizers, was similar to that of the guinea pig tests. The overall correct classification rate of human sensitizers and nonsensitizers was 61% (34/56) for the LLNA versus 59% (33/56) for the guinea pig tests. The LLNA correctly classified more strong sensitizers and other than strong sensitizers than did guinea pig tests; however, the LLNA correctly classified fewer nonsensitizers. The LLNA correctly classified 71% (10/14) of the strong human sensitizers versus 57% for the guinea pig tests and 67% (14/21) of the other human sensitizers versus 52% (11/21) for the guinea pig tests. The LLNA also correctly classified 48% (10/21) of the nonsensitizers versus 67% (14/21) for the guinea pig tests.

3.5 Test Method Reliability

3.5.1 Intra- and Interlaboratory Variability

Basketter and Cadby (2004) evaluated the intralaboratory variability associated with 29 individual EC3 values for isoeugenol. The EC3 values ranged from 0.5% to 2.6%. These data were used to support the "often-mentioned perspective that the biological variation associated with the estimation of EC3 values means that any particular EC3 value can be halved or doubled" (Basketter and Cadby 2004). Basketter et al. (2007) evaluated the interlaboratory reproducibility of EC3 data for 17 sensitizers tested in at least two laboratories using the same vehicle. The authors concluded that, although variability exists, it is less than an order of magnitude.

3.5.2 Influence of LLNA Vehicle

A number of analyses included in the BRD (**Appendix C**) highlight the potential impact of the LLNA vehicle on EC3 values and potency classification. Forty-five substances in the NICEATM LLNA database of over 600 substances had data from tests in multiple vehicles. Evaluation revealed that potency classifications differed for 18% (8/45) of these substances with the GHS classification system (e.g., the EC3 value would change from ≤2% to >2%, or vice versa). Nine percent (4/45) of these substances had EC3 values that varied by at least an order of magnitude depending on the vehicle used in the LLNA. Another 24% (11/45) of the substances were classified differently as either sensitizers or nonsensitizers depending on the vehicle. Additionally, there were instances in which LLNA results from the same vehicle produced discordant sensitizer and nonsensitizer outcomes (16% [7/45] of the substances).

Vehicle may be an important determinant of the EC3 value but perhaps not for every substance tested or for a particular group of substances. With respect to the accuracy analyses (see **Section 3.4**), two-way analyses of variance with chemical and vehicle as the factors indicated that two vehicles were responsible for a statistically significant effect of vehicle on the LLNA EC3 value, propylene glycol and Pluronic L92 (see **Appendix C, Annex IV**). Linear regression and Spearman correlation analyses (Steel and Torrie 1980) indicated that removing tests using these vehicles had no impact on the relationship of the EC3 value with human DSA_{05} values for the 63 substances that were sensitizers in the LLNA and in the HMT and/or HRIPT.

In the classification rate analyses (see **Section 3.4**), the variability of the LLNA EC3 values for sensitizers was similar to that of DSA_{05} values. For LLNA and human sensitizers, the coefficient of variation (CV) range for the LLNA EC3 values was 2% to 349%, and the CV range for the DSA_{05} values was 2% to 408%.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The proposal for using the LLNA to determine potency does not impact its requirement for using animals or the number of animals that are required. However, this application could broaden the use of the LLNA protocol in place of guinea pig tests and thereby further reduce the number of guinea pigs being used to assess skin sensitization potential. The LLNA test method protocol requires a minimum of only four mice per treatment group, whereas currently recommended guinea pig tests require at least 10 guinea pigs per group for the Buehler test and at least five guinea pigs per group for the guinea pig maximization test. The LLNA is also a refinement compared with guinea pig tests because it avoids the pain and distress that occur in guinea pigs when substances cause allergic contact dermatitis.

4.0 ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation of the use of the LLNA to determine skin sensitization potency included one public review meeting by an independent scientific peer review panel, comments from SACATM, and multiple opportunities for public comments (see **Sections 1.0** and **4.2**). ICCVAM and the interagency IWG considered the Panel report, the SACATM comments, and all public comments before finalizing the ICCVAM test method evaluation report and BRD. This section summarizes ICCVAM consideration of these reports and comments. The Panel report and public comments are provided in **Appendices D2** and **F2**, respectively.

4.1 ICCVAM Consideration of Independent Peer Review Panel Report

4.1.1 Comments on Draft ICCVAM Recommendations: Test Method Usefulness and Limitations

The Panel agreed with the ICCVAM draft recommendation made in January 2008 that the LLNA should not be considered as a stand-alone test method for determining skin sensitization potency but could instead be used as part of a weight-of-evidence evaluation (e.g., along with quantitative structure-activity relationship, peptide reactivity, human evidence). The Panel further stated that additional analyses suggested at the March 2008 Panel meeting might improve the correlation between the EC3 values and the human threshold values, thus providing more information on the usefulness of the LLNA for predicting skin sensitization potency categories. The Panel did note that the effect of vehicles should be recognized as a limitation in the data analyses and a likely source of within- and between-laboratory variability.

ICCVAM Response:
ICCVAM considered the Panel report and performed additional analyses to compare the EC3 values and alternative human threshold values. This exercise was reported in the final BRD (see **Appendix C, Section 6.1**). Based on these analyses, ICCVAM concluded that the LLNA could be used to categorize substances as strong sensitizers (GHS Subcategory 1A) when the estimated concentration that produces a positive LLNA result (i.e., EC3) is ≤2%. However, when the substance produces an LLNA EC3 > 2%, additional information is needed to categorize a substance as an other than strong sensitizer (GHS Subcategory 1B: "other" skin sensitizer) (see **Section 2.1**).

4.1.2 Comments on Draft ICCVAM Recommendations: Test Method Protocol

The Panel concurred with the ICCVAM draft recommendation made in January 2008 that the ICCVAM-recommended LLNA protocol should be used when generating data that might be considered for skin sensitization potency categorization. Furthermore, they supported the recommendation that data should always be collected from individual animals and not pooled. Some Panel members offered the opinion that pooled data (OECD 2002)[16] should also be considered acceptable. The Panel suggested that the calculation for the EC3 value be included as part of the LLNA protocol.

ICCVAM Response:
ICCVAM supports the Panel recommendation that the ICCVAM-recommended LLNA protocol should be used when generating data that might be considered for skin sensitization potency categorization. However, ICCVAM disagreed with the Panel minority with regard to the acceptability of pooled data. Rather, ICCVAM concluded that, if experiments are performed using the ICCVAM-

[16] Updated in 2010 (OECD 2010a).

recommended LLNA protocol, the lymph nodes should be collected individually for each mouse. This is necessary in order to identify whether any of the individual animal responses are outliers. The capacity to identify outliers will help avoid false negative results for weaker sensitizers (i.e., substances that normally would produce an SI just above 3 might be incorrectly classified as negative due to a low outlier value).

The updated ICCVAM-recommended LLNA test method protocol, **Appendix B**, provides a detailed description of the LLNA and describes the calculation of the SI, which is used to determine the sensitizing potential of a test substance. Calculation of the EC3 value, which is the metric for predicting skin sensitization potency using the LLNA, is also included in this updated ICCVAM-recommended LLNA test method protocol.

4.1.3 Comments on Revised Draft ICCVAM Recommendations: Future Studies

The Panel agreed with the ICCVAM draft recommendation made in January 2008 that more data are needed to determine the optimal threshold to distinguish between weak and strong skin sensitizers in humans. However, the Panel discouraged conducting new animal studies unless it was likely that results from such studies would lead to an overall reduction in animal use. The Panel stated further that the LLNA could be used in conjunction with quantitative-structure activity relationship information, guinea pig assays, HMT/HRIPT, and quantitative data for elicitation and frequency of positive response in humans in a weight-of-evidence approach.

The Panel also suggested additional evaluations that might improve the correlation between LLNA and human data (e.g., dividing LOEL by a safety factor other than 10, using LOEL data only, or using NOEL data only). One Panel member suggested that using the DSA_{05} value was a better comparison to the EC3 value because the DSA_{05} represented a LOEL that was corrected to 5% incidence of an induction response. The Panel further stated that LLNA tests based on pooled or individual animal data should be evaluated independently to assess the impact of using pooled data on the accuracy for determining skin sensitization potency. The Panel recommended a statistical analysis to determine where an appropriate cutoff value between weak or strong sensitizers might be best defined for traditional LLNA data. For example, receiver-operator characteristic curves (Fawcett 2006) could be used to identify the optimum cutoff for determining the difference between weak and strong sensitizers. Finally, the Panel stated that the effect of different vehicles should be recognized as a limitation in the current data analysis, that this was a source of variability within and between laboratories, and that its impact should be considered in future analyses.

ICCVAM Response:
ICCVAM considered the Panel report and noted its positions regarding (1) the conduct of new animal studies; (2) the use of the LLNA in conjunction with other available information, data, and assay results in a weight-of-evidence approach; and (3) the conduct of additional evaluations that might improve the correlation between LLNA and human data. Accordingly, ICCVAM performed additional analyses to compare the EC3 values and the human threshold values (**Appendix C, Section 6.1**). Based on these analyses, ICCVAM concluded that the LLNA could be used to categorize substances as strong skin sensitizers (GHS Subcategory 1A) when the estimated concentration that produces a positive LLNA result (i.e., EC3) is ≤2%. However, additional information is required to categorize a substance as an other than strong sensitizer (GHS Subcategory 1B: “other” skin sensitizer) when the substance produces an LLNA EC3 > 2% (see **Section 2.0**).

4.2 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process provides numerous opportunities for stakeholder involvement, including submitting written public comments and providing oral comments at ICCVAM independent peer review panel meetings and SACATM meetings. **Table 4-1** lists the seven opportunities for

public comment that were provided during the ICCVAM evaluation of the validation status of new versions and applications of the LLNA, which included assessing use of the LLNA to determine skin sensitization potency categories. The number of public comments received in response to each of the opportunities is also indicated. A total of 45 comments were submitted. Detailed comments received in response to or related to the *Federal Register* notices listed in **Table 4-1** are available on the NICEATM-ICCVAM website.[17] The following sections, delineated by *Federal Register* notice and public meeting, briefly discuss the public comments received.

Table 4-1 Opportunities for Public Comments

Opportunities for Public Comments	Date	Number of Public Comments
72 FR 27815: The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data	May 17, 2007	17
72 FR 52130: Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments	September 12, 2007	4
73 FR 1360: Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments	January 8, 2008	7
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	March 4-6, 2008	16
73 FR 25754: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	May 7, 2008	1
73 FR 29136: Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	May 20, 2008	0
SACATM Meeting, Radisson Hotel, RTP, NC	June 18-19, 2008	0

4.2.1 Public Comments in Response to 72 FR 27815 (May 17, 2007): The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

NICEATM requested the following:

1. Public comments on the appropriateness and relative priority of evaluation of the validation status of the following:
    a. The LLNA as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification
    b. The reduced LLNA approach (ESAC 2007; ICCVAM 2009; Kimber et al. 2006)
    c. Nonradioactive LLNA methods
    d. The use of the LLNA for testing mixtures, aqueous solutions, and metals
    e. The current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been determined to be useful)

[17] http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm

2. Nominations of expert scientists to consider as members of a possible independent scientific peer review panel
3. Submission of data for the LLNA and/or modified versions of the LLNA

NICEATM received 17 comments in response to this *Federal Register* notice. Six comments included additional data and information, while two others offered data and information upon request. Three commenters nominated four potential panelists for consideration. Three commenters suggested reference publications for consideration during the Panel evaluation. The nominees were included in the database of experts from which the Panel was selected. The data and suggested references were included in the ICCVAM draft review documents that were provided to the Panel for the March 2008 meeting.

Comment:
A commenter suggested rearranging the priority sequence of test method evaluation from most to least pressing: a, e, d, b, and c (see list above).

ICCVAM Response:
ICCVAM did not establish a relative priority for these activities because they were all considered to be high-priority activities. Accordingly, all LLNA-related activities described above were discussed at the March 2008 Panel meeting.

Comment:
One comment pertained to LLNA skin sensitization potency.

Acknowledging that the LLNA must be validated for determining skin sensitization potency for regulatory use, the commenter urged ICCVAM to take an abbreviated test validation approach. The commenter encouraged ICCVAM to spend its time and resources promoting the development and regulatory use of non-animal methods by engaging in integrated approaches to *in vitro* immunotoxicity.

ICCVAM Response:
Traditional regulatory test methods for skin sensitization (i.e., guinea pig maximization test, Buehler test, LLNA) have focused on “yes” or “no” determinations of skin sensitization hazard. In recent years, the LLNA has been proposed as an effective method for determining skin sensitization potency because of the dose-response information that is generated. ICCVAM evaluated the LLNA for potency use and concluded that the LLNA could be used to categorize substances as strong sensitizers (GHS Subcategory 1A) when the estimated concentration that produces a positive LLNA result (i.e., EC3) is ≤2%. However, additional information is required to categorize a substance as an other than strong sensitizer (GHS Subcategory 1B: “other” skin sensitizer) when the substance produces an LLNA EC3 > 2%.

The proposal for using the LLNA for potency determinations does not impact its requirement for using animals or the number of animals that will be required. However, this application could broaden the use of the LLNA protocol in place of guinea pig tests and could thereby further reduce the number of guinea pigs that are being used to assess skin sensitization potential. ICCVAM acknowledges the desire to abbreviate the validation approach and is committed to performing test method validations in the most scientifically expeditious and efficient manner possible. However, ICCVAM is also committed to promoting human safety and, accordingly, is dedicated to ensuring the relevance and reliability of alternative test methods that reduce, refine, or replace animals used for such safety analyses. Further, ICCVAM is also committed to identifying *in vitro* models and integrated non-animal approaches for assessing ACD. ICCVAM is engaged with ECVAM and JaCVAM in the development of validation studies for such methods. Timely regulatory adoption of properly vetted and thoroughly validated test methods is the desired consequence and sought-after goal of these international validation organizations.

4.2.2 Public Comments in Response to 72 FR 52130 (September 12, 2007): Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

NICEATM requested public comments on the September 2007 draft ICCVAM-recommended LLNA performance standards developed to facilitate evaluation of modified LLNA test method protocols. In response to this *Federal Register* notice, NICEATM received four comments.

None of the comments specifically addressed LLNA skin sensitization potency.

4.2.3 Public Comments in Response to 73 FR 1360 (January 8, 2008): Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

NICEATM requested public comments on the January 2008 draft BRDs, draft ICCVAM test recommendations, draft test method protocols, and revised draft LLNA performance standards for an independent international scientific peer review panel meeting held March 4-6, 2008, to evaluate modifications and new applications for the LLNA. NICEATM received 23 comments in response to this FR notice; seven written comments were received in advance of the meeting, and 16 oral comments were offered at the Panel meeting.

Public Comments, Written

Five written comments were relevant to LLNA skin sensitization potency. One commenter submitted two separate comments.

Comment:

The commenter acknowledged the considerable detail and information that was involved in this evaluation but indicated that human data on skin sensitization thresholds has been given undue status as an accurate gold standard and delineated a number of issues that are problematic for the human no effect and lowest effect threshold data.

ICCVAM Response:

Uncertainties with the human data are acknowledged (e.g., variable human protocols and results) and discussed in the BRD (**Appendix C, Section 4.0**). An analysis of variability for human and LLNA skin sensitizers indicated that the CV range for DSA_{05} values (2%-408%) was similar to that for LLNA EC3 values (2%-349%) (see **Appendix C, Section 7.0**). Despite the limitations of the human data, a positive and statistically significant correlation between LLNA and human data exists. A linear regression analysis of geometric mean human DSA_{05} on the geometric mean LLNA EC3 yielded $R^2 = 0.448$ with a statistically significant slope ($p < 0.0001$) (see **Appendix C, Section 6.1.1**). As suggested by a Panel member, the analysis used the DSA_{05} for the human threshold as a better comparison to the EC3 value because it represents an LOEL corrected to 5% incidence of an induction response. As improvements are made in standardizing the predictive human tests, the results can be considered with more certainty.

Comment:

A second commenter indicated that the approach by ICCVAM to validate the LLNA for the prediction of strong and weak skin sensitizers poses a methodological challenge. The commenter noted that it is possible that available HMT and HRIPT data may lead to a false human skin sensitization potency categorization because it is often difficult to correctly interpret the total dose used in the human tests due to insufficient documentation of total area dosed or prior patient exposure. The commenter further indicated that the criteria used to select the LLNA data used in the analyses should also be more thoroughly discussed (e.g., LLNA protocols and solvents, geometric

mean versus most conservative mean for substances with multiple studies, representation of substances in the LLNA database).

ICCVAM Response:
As noted above, uncertainties with the human data are acknowledged and discussed in the BRD (**Appendix C, Section 4.0**), and an analysis of variability for human and LLNA skin sensitizers indicated that the CV ranges for human and LLNA threshold values are similar (**Appendix C, Section 4.0**). Detail has been added on the calculations for the dose per unit area used in the human predictive tests, and the possibility of misclassification has been discussed. Such uncertainties did not prevent a statistically significant relationship of human DSA_{05} with the LLNA EC3 values ($R^2 = 0.448$ for the geometric mean linear regression; $p < 0.0001$ for slope) (see **Appendix C, Section 6.1.1**). The BRD also discusses numerous analyses performed by NICEATM to determine the optimal LLNA criteria for the current validation database. The geometric mean regression yielded $R^2 = 0.448$, and the most potent regression yielded $R^2 = 0.382$. The inclusion of LLNA results from different vehicles or from nonstandard protocols did not impact the relationship of the EC3 with the DSA_{05} values (see **Appendix C, Annex IV**).

Comment:
A third commenter observed the difficulties in comparing LLNA EC3 values to human data and to guinea pig data. The commenter also criticized the proposed classification categories for skin sensitization in the January 2008 draft BRD that use guinea pig tests for potency classification.

ICCVAM Response:
With regard to comparisons between LLNA EC3 values and human data, uncertainties with the human data are acknowledged (e.g., variable human protocols and results) and discussed in the BRD (**Appendix C, Section 4.0**). Such uncertainties did not prevent a statistically significant relationship of human DSA_{05} with the LLNA EC3 values ($R^2 = 0.448$ for the geometric mean linear regression; $p < 0.0001$ for slope) (see **Appendix C, Section 6.1.1**).

With regard to comparisons between LLNA EC3 values and guinea pig data, it is acknowledged in the BRD that the guinea pig tests are designed for hazard identification and are not well suited for potency estimations. However, the third revised edition of the GHS also includes criteria for sensitizer subcategories 1A ("strong" skin sensitizers) and 1B ("other" skin sensitizers) based on results from guinea pig tests (UN 2009). Since the January 2008 draft BRD, the analyses evaluating the accuracy of the LLNA to predict skin sensitization potency in guinea pigs have been removed from the evaluation. A comparison of the accuracy of the guinea pig outcomes to correctly classify human skin sensitization potency with the accuracy of the LLNA to correctly classify human skin sensitization potency has been retained (see **Appendix C, Section 6.2**). ICCVAM will continue to assess performance of new test methods against both the currently accepted test, as well as against existing human data and/or experience.

The proposed classification categories in the January 2008 draft BRD referred to by the commenter were finalized in the third revised edition of the GHS, which was recently adopted and published (UN 2009).

Public Comments, Oral
Two oral comments related to LLNA skin sensitization potency.

Comment:
One commenter stated that it might be difficult to split potency data into pooled and unpooled groups. This is because the majority of available data likely comes from pooled groups, and conclusions that individual animal data must be used were derived from analyses based primarily on pooled data from four animals.

The commenter expressed concern about human threshold data being considered as the gold standard for the comparative analyses. However, the commenter considers the analyses adequate for recommending the LLNA as a part of a weight-of-evidence decision on human skin sensitization potency categorizations.

ICCVAM Response:
The BRD discusses numerous analyses performed by NICEATM to determine the representative LLNA EC3 value for the substances in the validation database (see **Appendix C, Annex IV**). Analyses separating LLNA data, based on the collection of either individual animal or pooled data, have not been performed and could be considered as part of ICCVAM's continuing efforts to assess test method performance.

Uncertainties with the human data are acknowledged (e.g., variable human protocols and results) and discussed in the BRD (see **Appendix C, Section 4.0**), but these uncertainties did not prevent a statistically significant relationship of human DSA_{05} with the LLNA EC3 values ($R^2 = 0.448$ for the geometric mean linear regression; $p < 0.0001$ for slope) (see **Appendix C, Section 6.1.1**).

Comment:
Another commenter noted that there has been much discussion about various ways of handling the potency data. The OECD expert task force on skin sensitization needs to see an analytical comparison of what is considered the most appropriate approach for evaluating the data. The question for categorization purposes is: what is the ideal testing modality for separating strong versus weak sensitizers for potency categorization? A regulator who must assign a categorization is going to be confronted with all available test data and must know which data should be given the greatest weight in their evaluation.

The commenter indicated that the OECD task force also reviewed the January 2008 draft BRD on potency determinations and sent a list of several questions to the Panel. One of the questions is whether the LLNA protocols can be refined (e.g., by selection of solvents or choice of other test parameters) to improve correlation. The commenter concluded by expressing hope that the additional analyses that the Panel has suggested will bring some clarity to the matter.

ICCVAM Response:
NICEATM considered numerous comments from the public and the Panel in finalizing the analyses related to LLNA skin sensitization potency. The BRD includes numerous analyses performed by NICEATM to determine the optimal LLNA criteria for the current validation database (see **Appendix C, Annex IV**). For the substances in the validation database, LLNA vehicle did not have a significant impact on the relationship of the LLNA EC3 value to the human DSA_{05} values. Additionally, the inclusion of LLNA results (20% [132/653] of the LLNA tests used) from nonstandard LLNA protocols that used different mouse strains, both sexes of mice, different dosing schedules, different durations between the last topical application and the injection of radioactive marker, and pretreatment with sodium lauryl sulfate did not have a significant impact on the relationship of the LLNA EC3 values to the human DSA_{05} values.

4.2.4 Public Comments in Response to 73 FR 25754 (May 7, 2008): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics.

One public comment was received in response to this *Federal Register* notice, and it did not specifically address LLNA skin sensitization potency.

4.2.5 Public Comments in Response to 73 FR 29136 (May 20, 2008): Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested written public comments on the *Peer Review Panel Report*.

No public comments were received in response to this *Federal Register* notice.

4.2.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008

The June 18-19, 2008, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method (see **Appendix F3**).

No public comments related specifically to the topic of LLNA skin sensitization potency were offered.

Regarding LLNA skin sensitization potency, one SACATM member noted that the use of the LLNA for potency determinations was unclear and asked if this was for a validation study.

ICCVAM Response:
In 2007, the CPSC expressed concern to ICCVAM that the LLNA was being proposed internationally for use in potency determinations for the purpose of classification even though the LLNA had not undergone formal validation for this purpose. Thus, CPSC requested that NICEATM-ICCVAM assess the validation status of the LLNA as a stand-alone assay for potency determinations (including severity) for classification purposes.

5.0 References

Basketter DA, Cadby P. 2004. Reproducible prediction of contact allergenic potency using the local lymph node assay. Contact Dermatitis 50(1): 15-17.

Basketter DA, Gerberick F, Kimber I. 2007. The local lymph node assay and the assessment of relative potency: status of validation. Contact Dermatitis 57(2): 70-75.

Buehler EV. 1965. Delayed contact hypersensitivity in the guinea pig. Arch Dermatol 91: 171-177.

EPA. 2003. Health Effects Test Guidelines: OPPTS 870.2600 - Skin Sensitization. EPA 712-C-03-197. Washington, DC:U.S. Environmental Protection Agency. Available: http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf [accessed 17 September 2009].

ESAC. 2007. ESAC Statement on the Reduced Local Lymph Node Assay. Ispra, Italy:European Commission Directorate General, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods. Available: http://ecvam.jrc.it/ft_doc/ESAC26_statement_rLLNA_20070525-1.pdf [accessed 6 July 2010].

Fawcett T. 2006. An introduction to ROC analysis. Pattern Recognition Letters 27(8): 861-874.

Gerberick GF, Ryan CA, Kern PS, Dearman RJ, Kimber I, Patlewicz GY, et al. 2004. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. Contact Dermatitis 50(5): 274-288.

Hutchings CV, Shum KW, Gawkrodger DJ. 2001. Occupational contact dermatitis has an appreciable impact on quality of life. Contact Dermatitis 45(1): 17-20.

ICCVAM. 1999. The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds. The Results of an Independent Peer Review Evaluation Coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). NIH Publication No. 99-4494. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf [accessed 21 October 2010].

ICCVAM. 2001. ICCVAM Immunotoxicology Working Group Recommended Protocol for the Murine Local Lymph Node Assay (LLNA): Testing of Chemicals for Contact Sensitizing (Allergic Contact Dermatitis [ACD]) Potential. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf [accessed 29 November 2010].

ICCVAM. 2009. ICCVAM Test Method Evaluation Report. The Reduced Murine Local Lymph Node Assay: An Alternative Test Method Using Fewer Animals to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 09-6439. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNA-LD/TMER.pdf [accessed 21 October 2010].

ICCVAM. 2010a. ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: BrdU-ELISA. A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 10-7552. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/TMER.htm [accessed 21 October 2010].

ICCVAM. 2010b. ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA. A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis

Potential of Chemicals and Products. NIH Publication No. 10-7551. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/TMER.htm [accessed 21 October 2010].

Kimber I, Basketter DA, Berthold K, Butler M, Garrigue JL, Lea L, et al. 2001. Skin sensitization testing in potency and risk assessment. Toxicol Sci 59(2): 198-208.

Kimber I, Dearman RJ, Betts CJ, Gerberick GF, Ryan CA, Kern PS, et al. 2006. The local lymph node assay and skin sensitization: a cut-down screen to reduce animal requirements? Contact Dermatitis 54(4): 181-185.

Kligman AM. 1966. The identification of contact allergens by human assay. 3. The maximization test: a procedure for screening and rating contact sensitizers. J Invest Dermatol 47(5): 393-409.

Kligman AM, Epstein W. 1975. Updating the maximization test for identifying contact allergens. Contact Dermatitis 1(4): 231-239.

Magnusson B, Kligman AM. 1970. Allergic Contact Dermatitis in the Guinea Pig. Springfield, IL:Charles C. Thomas.

Marzulli FN, Maibach HI. 1974. The use of graded concentrations in studying skin sensitizers: experimental contact sensitization in man. Food Cosmet Toxicol 12(2): 219-227.

OECD. 1992. Test No. 406. Skin Sensitisation [adopted 17 July 1992]. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris:OECD Publishing. Available: http://dx.doi.org/10.1787/9789264070660-en [accessed 30 July 2010].

OECD. 2002. Test No. 429. Skin Sensitisation: Local Lymph Node Assay [adopted 24 April 2002]. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris:OECD Publishing. Available: http://dx.doi.org/10.1787/9789264071100-en [accessed 30 July 2010].

OECD. 2010a. Test No. 429. Skin Sensitization: Local Lymph Node Assay [adopted 22 July 2010]. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris:OECD Publishing. Available: http://dx.doi.org/10.1787/9789264071100-en [accessed 6 August 2010].

OECD. 2010b. Test No. 442A. Skin Sensitization: Local Lymph Node Assay: DA [adopted 22 July 2010]. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris:OECD Publishing. Available: http://dx.doi.org/10.1787/9789264090972-en [accessed 6 August 2010].

OECD. 2010c. Test No. 442B. Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA [adopted 22 July 2010]. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris:OECD Publishing. Available: http://dx.doi.org/10.1787/9789264090996-en [accessed 6 August 2010].

Politano VT, Api AM. 2008. The Research Institute for Fragrance Materials' human repeated insult patch test protocol. Regul Toxicol Pharmacol 52(1): 35-38.

Skoet R, Zachariae R, Agner T. 2003. Contact dermatitis and quality of life: a structured review of the literature. Br J Dermatol 149(3): 452-456.

Steel RGD, Torrie JH. 1980. Principles and Procedures of Statistics A Biometrical Approach. New York:McGraw Hill.

UN. 2009. Globally Harmonized System for Classification and Labelling of Chemicals. 3rd rev. ed. New York:United Nations. Available: http://www.unece.org/trans/danger/publi/ghs/ghs_rev03/03files_e.html [accessed 21 October 2010].