ICCVAM Test Method Evaluation Report on Using the Murine Local Lymph Node Assay for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

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
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List of Abbreviations and Acronyms

AOO	Acetone: olive oil (4:1 by volume)
BAuA	Federal Institute for Occupational Safety and Health (Germany)
BRD	Background review document
BT	Buehler test
CPSC	U.S. Consumer Product Safety Commission
DMSO	Dimethyl sulfoxide
DNCB	Dinitrochlorobenzene
EC3	Estimated concentration needed to produce a stimulation index of 3
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FR	*Federal Register*
GP	Guinea pig
GPMT	Guinea pig maximization test
HCA	Hexyl cinnamic aldehyde
HSUS	Humane Society of the United States
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
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organisation for Economic Co-operation and Development
P.L.	Public Law
rLLNA	Reduced murine local lymph node assay
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
U.S.C.	United States Code

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[2] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Updated 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.*

[3] Dr. Richmond was unable to attend the public meeting on March 4-6, 2008. However, he was 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.*

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Preface

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin-sensitizing chemicals and products. ACD results in lost workdays[1] 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. Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary 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 and recommended an alternative known as the murine (mouse) local lymph node assay ("traditional LLNA").[2] The traditional 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. 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 traditional LLNA as a valid alternative test method for ACD testing.

In 2007, the U.S. Consumer Product Safety Commission requested that ICCVAM evaluate the usefulness and limitations of the LLNA for testing mixtures, metals, and substances in aqueous solutions (i.e., an evaluation of the current applicability domain of the LLNA), among other activities related to the LLNA. ICCVAM assigned this activity a high priority after considering comments from the public and ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). As part of their ongoing collaboration with ICCVAM, scientists from the European Centre for Validation of Alternative Methods (ECVAM) and the Japanese Center for Validation of Alternative Methods (JaCVAM) served as liaisons to the ICCVAM Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA applicability domain evaluation is included with this report.

This test method evaluation report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA for assessing the ACD potential of pesticide formulations, metals, substances tested in aqueous solutions, and other products. The report also provides the updated ICCVAM-recommended LLNA test method protocol. The database of substances used to evaluate the current applicability domain of the LLNA is discussed and summarized.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the evaluation process. ICCVAM considered the SACATM comments, the Independent Scientific Peer Review Panel's report, and all public comments before finalizing this ICCVAM Test Method Evaluation Report. The ICCVAM Test Method Evaluation Report will be provided to U.S. Federal regulatory agencies for consideration and be made available to the public. The ICCVAM Authorization Act requires that Federal agencies respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. Agency responses will be posted on the NICEATM-ICCVAM website[3] as they become available.

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

[1] http://www.blf.gov/IIF

[2] The "traditional LLNA" refers to the validated ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxy-uridine into the cells of the draining auricular lymph nodes (ICCVAM 1999, Dean et al. 2000).

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

serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Dr. Stephen Ullrich, and Kim Headrick for their service as Evaluation Group Chairs. We thank the IWG for assuring a meaningful and comprehensive review. We especially thank Dr. Joanna Matheson (U.S. Consumer Product Safety Commission) and Dr. Abigail Jacobs (U.S. Food and Drug Administration Center for Drug Evaluation and Research) for serving as Co-Chairs of the IWG. We also acknowledge Integrated Laboratory Systems, Inc. (ILS), 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 Dr. Silvia Casati and Dr. Hajime Kojima, the IWG liaisons from ECVAM and JaCVAM, respectively, for their participation and contributions.

This comprehensive ICCVAM evaluation of the LLNA applicability domain should facilitate regulatory agency decisions on the acceptability of the LLNA for evaluating the allergic contact dermatitis potential of pesticide formulations, metals, substances tested in aqueous solutions, and other products. Use of the method by industry can be expected to significantly reduce and refine animal use for ACD testing while continuing to support the protection of human health.

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Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the applicability domain of the murine local lymph node assay (LLNA). Applicability domain refers to defined chemicals and products for which a test method can be used to obtain accurate and reliable results. The LLNA assesses the potential of substances to cause allergic contact dermatitis (ACD). ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. This Test Method Evaluation Report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA for testing pesticide formulations, metals, substances in aqueous solutions, and other products (i.e., the current applicability domain of the LLNA). This report includes the updated ICCVAM-recommended LLNA test method protocol, the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999), and recommendations for future studies and performance standards.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and the ICCVAM Immunotoxicity Working Group prepared an initial draft Addendum and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (Panel) and the public for comment. The initial draft Addendum reviewed LLNA data from a database of more than 500 test substances. It built on the original ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). The Panel met twice in public session to review the initial and updated draft Addendums and draft ICCVAM recommendations. A detailed timeline of the evaluation of the LLNA applicability domain is included with this report.

The Panel initially met in public session on March 4–6, 2008, to discuss its peer review of the ICCVAM initial draft Addendum and to provide conclusions and recommendations regarding the LLNA applicability domain. The Panel also reviewed how well the information contained in the initial draft Addendum supported ICCVAM's draft test method recommendations. The Panel agreed with ICCVAM that the LLNA appeared useful for the testing of metal compounds, with the exception of nickel. The Panel agreed with the ICCVAM recommendations, which stated that more data were necessary before a recommendation could be made on the usefulness and limitations of the LLNA for testing mixtures and substances in aqueous solutions.

NICEATM obtained the additional data and updated the initial draft Addendum. The updated draft Addendum evaluated data derived from a database of more than 600 substances tested in the LLNA (including pesticide formulations and other products). The Panel reconvened in public session on April 28–29, 2009, to review the ICCVAM updated draft Addendum and to finalize its conclusions and recommendations on the current LLNA applicability domain. In finalizing this Test Method Evaluation Report and the Addendum, which is included as an appendix, 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 accuracy performance of the LLNA supports its use for testing (1) pesticide formulations and other products; (2) metals, with the exception of nickel; (3) substances tested in aqueous solutions; and (4) other products and substances, unless these materials have unique physiochemical properties associated with them that might interfere with the LLNA's ability to detect sensitizing substances. To achieve adequate exposure, substances in aqueous solutions must be tested in an appropriate vehicle (e.g., 1% Pluronic L92 [Boverhoff et al. 2008]) that will maintain adequate contact of the test substance with the skin. The determination that a specific modification of the LLNA test method protocol is valid for evaluating new chemical classes should be relevant to other valid versions of the LLNA test method protocol (e.g., LLNA: DA and LLNA: BrdU-ELISA).

As shown in **Table 1**, the LLNA is more likely than the guinea pig test to yield a positive result for many substances. Therefore, the potential for overclassification may be a limitation of the LLNA. Federal agencies should assess how well the test materials and findings in the updated draft Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

ICCVAM Recommendations: Test Method Protocol

ICCVAM recently updated the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a). ICCVAM recommends this revised protocol for all future LLNA studies.

Additionally, in testing situations that do not require dose-response information, the LLNA should be considered as a reduced LLNA test method protocol. The reduced LLNA tests only the high dose, further reducing animal use.

ICCVAM Recommendations: Future Studies

ICCVAM recommends several future studies to further characterize the usefulness and limitations of the LLNA. However, ICCVAM discourages formal validation of the LLNA for new classes/types of test substances unless there is a biologically-based rationale. An integrated assessment of available information, including computer-assisted structure–activity relationships, prediction/measurement of biotransformation to potential reactive species, and possibly peptide, protein, or lipid binding should be conducted for new classes of test materials. Before any animal testing is conducted, the need to test a substance for skin sensitization potential should be considered.

Table 1 Summary of LLNA Performance for Testing Pesticide Formulations and Other Products, Metal Compounds, and Substances in Aqueous Solutions

Comparison	n	Accuracy		False Positive Rate		False Negative Rate	
		%	No.	%	No.	%	No.
Pesticide Formulations							
LLNA vs. GP[1]	23	57	13/23	50	10/20	0	0/3
Dyes							
LLNA vs. GP[1]	6	33	2/6	100	1/1	60	3/5
Natural Complex Substances							
LLNA vs. Human[2]	12	42	5/12	75	6/8	25	1/4
Metal Compounds							
LLNA vs. GP[1]	6	83	5/6	100	1/1	0	0/5
LLNA vs. Human[2]	14	86	12/14	40	2/5	0	0/9
Substances Tested in Aqueous Solutions							
LLNA vs. GP[1]	25	56	14/25	48	10/21	25	1/4

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; n = number of substances included in this analysis; No. = number (data on which the percentage calculation is based).
Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method; false positive rate = the proportion of all negative substances that are falsely identified as positive; false negative rate = the proportion of all positive substances that are falsely identified as negative.
[1] *GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
[2] *Human* refers to outcomes obtained by studies conducted using the human maximization test or a human patch test allergen kit.

ICCVAM Recommendations: Performance Standards

ICCVAM, the European Centre for the Validation of Alternative Methods, and the Japanese Center for the Validation of Alternative Methods have developed internationally harmonized test method performance standards for the LLNA (ICCVAM 2009a).[4] These performance standards can be used to evaluate the validity of LLNA test methods that incorporate specific modifications of the traditional LLNA test method.

Validation Status of the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

The Addendum summarizes information from a review of LLNA data derived from a database of more than 600 substances (including pesticide formulations and other products). It builds on the 1998-99 ICCVAM evaluation of the LLNA (ICCVAM 1999) that considered a database of 209 substances. To minimize duplication, metal formulations were not analyzed, and metal compounds were restricted to those testing single substances. The updated reference database includes (1) data for metal compounds from the original ICCVAM evaluation, (2) data published since that evaluation, and (3) data submitted in response to a *Federal Register* notice (72 FR 27815)[5] requesting LLNA, guinea pig, and/or human skin sensitization data and experience.

Pesticide Formulations: The updated LLNA database contains data for 104 pesticide formulations. Fifty-four percent of these formulations were LLNA positive, and 46% were LLNA negative.

Twenty-three pesticide formulations had associated guinea pig data for the complete formulation. An additional 46 formulations had guinea pig data for one or more of the active ingredients included in the formulation tested in the LLNA. Fourteen formulations had guinea pig data for a substance related to an active ingredient or for a related formulation.

Among the 23 formulations that had both LLNA and guinea pig data, the LLNA classified 52% (12 of 23) as sensitizers while the guinea pig tests classified 13% (3 of 23) as sensitizers. All three pesticide formulations identified as sensitizers in the guinea pig test were also identified as sensitizers in the LLNA. Overall, the LLNA and the guinea pig results had 57% agreement (accuracy) in 13 of 23 tests (**Table 1**). The LLNA identified as sensitizers an additional seven formulations that the guinea pig test classified as nonsensitizers, a possible overprediction (false positive) rate of 50% (10 of 20) (**Table 1**). However, human data were not available for these pesticide formulations to confirm their sensitization potential in humans.

Dyes: The current LLNA database contains data for six dyes that have comparative LLNA and guinea pig data. The LLNA classified 50% of the dyes as sensitizers and 50% as nonsensitizers. By comparison, the guinea pig maximization test (GPMT) classified 83% as sensitizers and 17% as nonsensitizers. Overall, the LLNA and GPMT results had 33% accuracy (**Table 1**). The overprediction (false positive) rate for the LLNA was 100% (1 of 1), and the underprediction (false negative) rate was 60% (3 of 5) (**Table 1**).

Natural Complex Substances: The current LLNA database contains data for 12 natural complex substances (essential oils and absolutes) with comparative LLNA and human data. The LLNA classified 75% (9 of 12) of these substances as sensitizers and 25% (3 of 12) as nonsensitizers. However, human clinical studies identified only 33% (4 of 12) as sensitizers. The LLNA identified three of these four as sensitizers (75%), but six more tested positive that did not produce positive results in the human testing. Compared to human outcomes, the LLNA had an accuracy of 42% (5 of 12), a false positive rate of 75% (6 of 8), and a false negative rate of 25% (1 of 4) (**Table 1**).

[4] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.
[5] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf.

Metal Compounds: The current LLNA database contains test results from 48 studies of 16 metal compounds. The compounds represent 13 different metals. (Formulations containing metals were excluded from this analysis.) All 16 metal compounds had comparative human data, and eight had comparative guinea pig data. Because nickel was classified as a sensitizer in three of seven studies and as a nonsensitizer in four of seven studies, nickel compounds were excluded from the LLNA metals performance analysis.

For the remaining 14 metal compounds (13 metals), the LLNA had an accuracy of 86% (12 of 14), a false positive rate of 40% (2 of 5), and a false negative rate of 0% (0 of 9) when compared to human results (**Table 1**). The two false positive compounds were copper chloride and zinc sulfate.

The LLNA classified as sensitizers all six of the metal compounds with comparative guinea pig test results (six different metals with nickel compounds excluded). For these metal compounds, the LLNA had an accuracy of 83%, a false positive rate of 100%, and a false negative rate of 0% (**Table 1**) when compared to guinea pig test results.

The performance of the LLNA and the guinea pig tests was compared to human results for the six metal compounds tested in all three species. The LLNA had accuracy of 83%, a false positive rate of 100%, and a false negative rate of 0%. By comparison, the guinea pig tests had an accuracy of 100%, a false positive rate of 0%, and a false negative rate of 0% relative to the human outcomes.

Substances Tested in Aqueous Solutions: The current LLNA database of substances tested in aqueous solutions includes results from 171 studies representing 139 substances. Ninety-one percent of these substances (123 LLNA studies) are pesticide formulations and pure compounds. Forty-eight percent (48 LLNA studies) are aqueous eluates of medical devices. The two groups were analyzed separately because of differences in the protocols for sample preparation. Of the 91 pesticide formulations and pure compounds, 63% (57 of 91) were LLNA positive, and 37% (34 of 91) were LLNA negative. The substances included in this evaluation were tested at a final concentration of at least 20% water.

Guinea pig data were available for 25 substances tested in aqueous solutions. The LLNA and the guinea pig test results disagreed for 11 (44%) of the substances. Ten of the 11 discordant substances (91%) were pesticide formulations tested in aqueous 1% Pluronic L92. These were the same 10 substances previously discussed for the pesticide formulations analysis. The LLNA overpredicted all 10 with respect to the guinea pig results (48% [10 of 21] false positive rate) (**Table 1**). The LLNA underpredicted one additional substance, neomycin sulfate, which was tested in 25% EtOH (25% [1 of 4] false negative rate) (**Table 1**). The LLNA and guinea pig results had overall agreement (accuracy) of 56% (14/25) (**Table 1**).

All 48 of the medical device eluates were negative in the LLNA. These eluates were not analyzed to determine their constituents or to determine whether any compound(s) were in fact eluted from the medical device tested.

ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process provides numerous opportunities for stakeholder involvement. The public may submit written comments and provide oral comments at ICCVAM independent scientific peer review panel meetings and SACATM meetings. From May 2007 to June 2009, there were a total of 12 opportunities for public comment on the ICCVAM evaluation of the LLNA applicability domain. During this time, ICCVAM received 46 public comments, nine of which pertained directly to the LLNA applicability domain. In addition, SACATM reviewed and commented on the draft ICCVAM recommendations and associated conclusions of the Panel during their annual meetings in June 2008 and June 2009. ICCVAM considered both public and SACATM comments in finalizing the test method recommendations provided in this report.