ICCVAM Recommendations for Use of the LLNA for Evaluating the Allergic Contact Dermatitis Potential of Pesticide Formulations and Other Products

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Abstract

ICCVAM has updated its 1999 validation report on the LLNA based on a recent evaluation of the usefulness and limitations of the LLNA for assessing the skin sensitizing potential of pesticide formulations. This review was initiated because the original report did not include an analysis of the LLNA for these types of substances, and there were growing regulatory concerns that the LLNA might not identify sensitizing pesticide formulations. LLNA data from 104 formulations were included in the evaluation, most of which are water soluble and therefore were tested in an aqueous vehicle containing 1% Pluronic L92. Of the pesticide formulations for which LLNA and guinea pig data were available (n=23), the LLNA classified 52% (12/23) as sensitizers, while GP tests classified only 13% (3/23) as sensitizers. All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA; there were no instances of underprediction by the LLNA. Thus, there appears a greater likelihood of obtaining a positive result in the LLNA than in a GP test. These studies also provide data for aqueous solutions that emphasize the need for careful selection of an appropriate vehicle that maintains test substance contact with the skin (e.g., 1% Pluronic L92 in water) to achieve adequate exposure when testing such substances. Based on these data, ICCVAM agreed with an international peer review panel that the LLNA could be used for testing pesticide formulations, and products in aqueous vehicles, unless there are physicochemical properties that may interfere with the ability of the LLNA to detect the sensitizing potential of a substance. ICCVAM recommendations will be forwarded to Federal agencies for regulatory acceptance consideration. Adoption of these recommendations should expand the use of the LLNA for skin sensitization testing, thereby reducing and refining animal use for this purpose.

Introduction

- The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is charged with evaluating the scientific validity of new, revised, and alternative toxicological test methods applicable to U.S. Federal agency safety testing requirements (Sailstad, et al., 2001).
  - ICCVAM forwards recommendations to Federal agencies.
  - By law, the agencies must respond to ICCVAM within 180 days.
- In response to a nomination by the U.S. Consumer Product Safety Commission in 2007, ICCVAM evaluated the applicability domain of the murine local lymph node assay (LLNA), a test method for assessing 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 repeated contact with a sensitizing chemical or product.
- ICCVAM's recommendations regarding the use of the LLNA for testing pesticide formulations and other products, metals, and substances in aqueous solutions (i.e., the current applicability domain of the LLNA) are documented in a Test Method Evaluation Report (TMER).
  - The ICCVAM TMER includes recommendations regarding:
    - Current usefulness and limitations of the LLNA
    - An LLNA test method protocol
    - Future studies
- The information summarized in this poster is based on a retrospective review of LLNA data derived from a database of over 600 substances (including pesticide formulations and other products) and builds on the 1998 ICCVAM evaluation of the LLNA (ICCVAM 1999; Dean et al.,2001; Haneke et al., 2001), which considered LLNA data for 211 substances.
- **Table 1** shows LLNA accuracy statistics compared to guinea pig and human results for the products and substances considered in this evaluation, which were derived from the database described above.
- The remainder of this poster will focus on the evaluation of pesticide formulations and substances tested in aqueous solutions.

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

Comparison LLNA vs. Reference Test Method Results	n[1]	Accuracy		Sensitivity		LLNA False Negative Rate		Specificity		LLNA False Positive Rate	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
Substances Tested in Aqueous Solutions											
LLNA vs. GP[3]	25	56	14/25	75	3/4	25	1/4	52	11/21	48	10/21
Pesticide Formulations											
LLNA vs. GP[3]	23	57	13/23	100	3/3	0	0/3	50	10/20	50	10/20
Metal Compounds											
LLNA vs. Human[4]	14	86	12/14	100	9/9	0	0/9	60	3/5	40	2/5
LLNA vs. GP[3]	6	83	5/6	100	5/5	0	0/5	0	0/1	100	1/1
Natural Complex Substances											
LLNA vs. Human[4]	12	42	5/12	75	3/4	25	1/4	25	2/8	75	6/8
Dyes											
LLNA vs. GP[3]	6	33	2/6	40	2/5	60	3/5	0	0/1	100	1/1

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; No. = number.
Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method;
Sensitivity = the proportion of all positive substances based on results from the reference test method (i.e., guinea pig or human testing/experience) that are classified as positive in the test method under evaluation (i.e. LLNA);
False negative rate = the proportion of all positive substances based on results in the reference test method (i.e., guinea pig or human testing/experience) that are identified as negative in the test method under evaluation (i.e. LLNA);
Specificity = the proportion of all negative substances based on results from the reference test method (i.e., guinea pig or human testing/experience) that are classified as negative in the test method under evaluation (i.e. LLNA);
False positive rate = the proportion of all negative substances based on results from the reference test method (i.e., guinea pig or human testing/experience) that are identified as positive in the test method under evaluation (i.e. LLNA)

[1] n = Number of substances included in this analysis.

[2] The data on which the percentage calculation is based.

[3] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4] *Human* refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

Validation Status of the LLNA for Testing:

1. Substances Tested in Aqueous Solutions

- The LLNA database included 171 studies representing 139 substances.
  - The substances were tested in the LLNA at a final concentration of at least 20% water
  - 91 substances (123 LLNA studies) were pesticide formulations and pure compounds.
  - 75 substances were pesticides tested in aqueous 1% Pluronic L92.
  - 48 substances (48 LLNA studies) were aqueous eluates of medical devices
- GP data were available for 25 substances tested in aqueous solutions.
  - The LLNA and the GP results were in agreement (accuracy) 56% (14/25) of the time (**Table 1**).
  - 11 substances were discordant between the LLNA and the GP tests.
  - 10/11 discordant substances were pesticide formulations tested in aqueous 1% Pluronic L92; these were the same 10 substances discussed for the pesticide formulations analysis, and all were overpredicted by the LLNA with respect to the GP results (48% [10/21] false positive rate) (**Table 1**).
    - 34% (25/75) pesticide formulations tested in aqueous 1% Pluronic L92 produced negative results in the LLNA.
    - Neomycin sulfate, tested in 25% Ethanol, was underpredicted by the LLNA with respect to the GP (25% [1/4] false negative rate) (**Table 1**).
- Because of sample preparation differences between the pesticide formulations and pure compounds, and medical device eluates, these groups were analyzed separately.
  - All 48 medical device eluates were LLNA negative.
  - These eluates were not analyzed to determine their constituents, or whether any compound(s) were eluted from the medical devices.

2. Pesticide Formulations

- The updated LLNA database included data for 104 pesticide formulations.
- 23 formulations had LLNA and GP data for the same formulation.
- There were no human skin sensitization test data or post-marketing sensitization report data.
- For the 23 formulations with both GP and LLNA data:
  - LLNA and the GP results were in agreement (accuracy) 57% (13/23) of the time (**Table 1**).
  - All 3 pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA.
  - The LLNA classified 52% (12/23) of formulations as sensitizers while GP tests classified 13% (3/23) as sensitizers.
  - The LLNA identified 7 additional substances as sensitizers that were classified as nonsensitizers in GP tests, an overprediction (i.e., false positive) rate of 50% (10/20) (**Table 1**).
  - No pesticide formulations were underpredicted (i.e., false negative) by the LLNA compared to the guinea pig results.

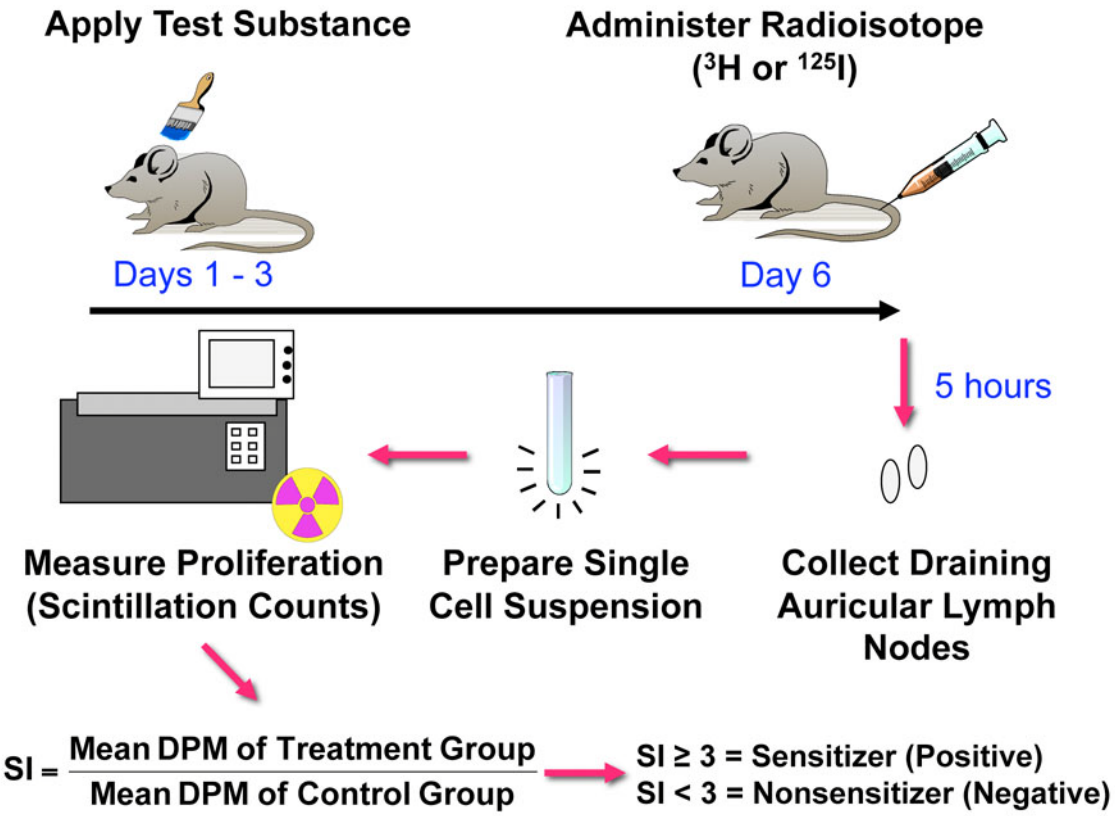
ICCVAM Recommendations: Test Method Usefulness and Limitations

- ICCVAM concludes that these data support the usefulness of the LLNA for testing pesticide formulations and other products, metals (with the exception of nickel), and substances tested in aqueous solutions, unless there are unique physiochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances.
  - When testing aqueous formulations in the LLNA, an appropriate vehicle should be added to prevent the test material from running off the skin (e.g. added pluronic acid to achieve1% Pluronic L92 [Boverhoff et al. 2008]) so an adequate dermal exposure is achieved.
  - If an LLNA variant (e.g., a nonradioactive LLNA version) is validated for use to test novel substance classes, then the findings should be relevant to the family of validated and accepted LLNA tests.
  - As indicated in **Table 1**, for many substances, there is a greater likelihood of obtaining a positive result in the LLNA than in a GP test. Therefore, the potential for possible overclassification may be a limitation of the LLNA.
  - Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

ICCVAM Recommendations: Test Method Protocol

- ICCVAM recommends that the updated LLNA test method protocol (Appendix A ,ICCVAM 2009a) should be used for all future LLNA studies, as it reduces animal use by 20% compared to the 1999 ICCVAM recommended protocol. **Figure 1** shows a schematic of the ICCVAM-recommended LLNA test method protocol.
- If no dose-response information is required or there is no basis to believe that the test article may be a sensitizer, a reduced LLNA test method protocol (testing only the high dose) should be considered, which will further reduce animal use by up to 40%. (ICCVAM 2009b)

Figure 1: Schematic of LLNA test method protocol



ICCVAM Recommendations: Future Studies

- ICCVAM recommended future studies include:
  - To more comprehensively evaluate the ability of the LLNA to be used for testing nickel compounds, additional data from LLNA studies on such compounds with comparative human and/or GP data are needed.
  - Available solubility data should be provided so that thermodynamic activity can be computed and compared to maximum theoretical percutaneous penetration.
    - Consider this information when comparing LLNA data from studies in lipophilic delivery systems vs. aqueous systems.
  - Use 1% Pluronic L92 in water as the vehicle for aqueous formulations in order to expand the existing database for that vehicle, unless adequate scientific rationale is provided for using another aqueous vehicle.
  - For new classes of test materials, conduct an integrated assessment of available information, including:
    - Computer-assisted structure-activity relationships
    - Prediction/measurement of biotransformation to potential reactive species
    - Possibly peptide, protein, or lipid binding.
  - While recommending future studies, ICCVAM emphasizes avoidance of revalidation of the LLNA for new classes/types of test substances unless a biologically-based rationale exists.
  - Before conducting animal testing, consider the necessity for the substance to be tested for skin sensitization potential.

ICCVAM Recommendations: Performance Standards

- In conjunction with ECVAM and JaCVAM, ICCVAM has developed internationally harmonized test method performance standards for the LLNA (ICCVAM 2009) to evaluate the performance of LLNA test methods that incorporate specific protocol modifications (e.g., procedures to measure lymphocyte proliferation) compared to the traditional LLNA.
- Final transmittal of these recommendations to agencies is currently in process.

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Timeline for the ICCVAM Evaluation of the LLNA Applicability Domain

Date	Event
January 10, 2007	CPSC nominates six LLNA review activities for ICCVAM evaluation,[1] including the LLNA applicability domain.
January 2007	ICCVAM IWG is re-established to work with NICEATM to carry out LLNA evaluations.
January 24, 2007	ICCVAM endorses the CPSC-nominated LLNA review activities
May 17, 2007	*Federal Register* notice (72 FR 27815) – *The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data*
June 12, 2007	SACATM endorses with high priority the six CPSC-nominated LLNA review activities
January 8, 2008	*Federal Register* notice (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*
March 4–6, 2008	International Independent Scientific Peer Review Panel convenes in public session with opportunity for oral public comments, at CPSC Headquarters in Bethesda, MD, to review new versions and applications of the LLNA
May 20, 2008	*Federal Register* notice (73 FR 29136) – *Announcement of the 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*[2]
June 18–19, 2008	SACATM public meeting: comments on the 2008 Panel report
February 27, 2009	*Federal Register* notice (74 FR 8974) – *Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments*
April 28–29, 2009	International Independent Scientific Peer Review Panel convenes in public session with opportunity for oral public comments, at NIH Natcher Conference Center in Bethesda, MD, to review new versions and applications of the LLNA
June 1, 2009	*Federal Register* notice (74 FR 26242) – *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: Notice of Availability and Request for Public Comments*[3]
June 25–26, 2009	SACATM public meeting: comments on the 2009 Panel report
October 28, 2009	ICCVAM endorses TMER for the LLNA applicability domain, which includes LLNA Addendum on the validity of the LLNA for mixtures, metals, and aqueous solutions.

Abbreviations: BRD = Background Review Document; CPSC = U.S. Consumer Product Safety Commission; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; IWG = ICCVAM Immunotoxicity Working Group; LLNA = Murine Local Lymph Node Assay; NICEATM = National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods; NIH = National Institutes of Health; SACATM = Scientific Advisory Committee on Alternative Toxicological Methods; TMER = Test Method Evaluation Report

[1]The CPSC nomination may be viewed on the NICEATM-ICCVAM website at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

[2] The report of the 2008 Peer Review Panel meeting is available at: http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

[3] The report of the 2009 Peer Review Panel meeting is available at:
http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf

Final transmittal of these recommendations to agencies is currently in process.

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LLNA Peer Review Panel Meetings

- Public meetings of an international independent scientific peer review panel ("Panel") organized by the ICCVAM and NICEATM were held at the Consumer Product Safety Commission in Bethesda, MD, on March 4-6, 2008, and at the National Institutes of Health in Bethesda, MD, on April 28-29,2009.

Charge to the Peer Review Panel

- Review the Addendum for errors and omissions.
- Provide conclusions and recommendations on the current validation status of the LLNA applicability domain.
- Does the information contained in the draft Addendum support ICCVAM's draft test method recommendations?

Peer Review Panel Conclusions

- The Panel concurred that that the data supported the ICCVAM Test Method Recommendations for LLNA usefulness and limitations.
- The Panel considered all of the test materials as candidates for testing in the LLNA, subject to the limitations outlined in the ICCVAM Test Method Recommendations.
- The Panel concluded that updated information did not suggest the need for changes to recommendations for the development of a revised standard method.
- At the discretion of the testers, the Panel recommended the inclusion of a suitable (representative) positive control from the same category of materials to be tested (e.g., for testing pesticides, select one representative positive control pesticide).
- The Panel concurred with ICCVAM's recommendations for future studies, and concurred that, before additional animal testing is conducted, consideration should be given to the necessity for the substance to be tested for skin sensitization potential.
- The complete LLNA Peer Review Panel Reports can be accessed at:
  - http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
  - http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf

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