ICCVAM Test Method Recommendations for the Reduced LLNA (rLLNA): An Alternative Test Method Using Fewer Animals to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

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Abstract

Based on recommendations by ICCVAM in 1999, U.S. regulatory agencies that require the submission of skin sensitization data accepted the LLNA, with identified limitations, as an alternative to guinea pig tests for assessing allergic contact dermatitis (ACD). In January 2007, the CPSC nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM. One of the nominated activities was an assessment of the usefulness and limitations of the rLLNA. In the rLLNA, each substance is tested at one dose level only (the high dose), whereas in the traditional LLNA, a minimum of three dose levels is tested. NICEATM and ICCVAM conducted a retrospective review of traditional LLNA data from 11 different sources that included 457 unique substances tested in 471 traditional LLNA studies. The ability of the rLLNA to correctly identify potential skin sensitizers was compared to traditional LLNA results. Based on the available data, the rLLNA has an accuracy of 99% (465/471), a false positive rate of 0% (0/153), and a false negative rate of 2% (6/318) when compared to the traditional LLNA. Based on these data, ICCVAM concluded that the rLLNA is sufficiently accurate to distinguish between skin sensitizers and non-sensitizers. Therefore, ICCVAM recommends that the rLLNA test method should be routinely used for determining the ACD potential of chemicals and products. ICCVAM has also made recommendations for a standardized rLLNA protocol, future studies to potentially improve the usefulness and applicability of the rLLNA, and the use of LLNA performance standards for modified rLLNA test methods. The comprehensive ICCVAM evaluation of the rLLNA should facilitate regulatory agency decisions on the acceptability of the method. Use of the method by industry can then be expected to significantly reduce animal use for ACD testing while continuing to support the protection of human health.

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Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is charged by the ICCVAM Authorization Act of 2000 with evaluating the scientific validity of new, revised, and alternative toxicological test methods applicable to U.S. Federal agency safety testing requirements. ICCVAM recently evaluated the validation status of the reduced murine local lymph node assay (rLLNA), 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 contact with a sensitizing chemical or product. ICCVAM's recommendations regarding the usefulness and limitations of the rLLNA as an alternative to the traditional murine local lymph node assay (LLNA) are documented in the Test Method Evaluation Report (TMER). The report also includes an updated ICCVAM-recommended LLNA test method protocol, recommendations for future studies, and the final rLLNA background review document (BRD). When deemed appropriate for use (e.g., if dose response information is not needed), the rLLNA can reduce by 40% the number of animals used for each test compared to the traditional LLNA.

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The rLLNA Test Method

The only difference between the test method protocols for the traditional LLNA and the rLLNA is the number of dose levels tested for a test substance.

- In the traditional LLNA, at least three dose levels are tested for each substance, with the highest dose based on maximum solubility and the avoidance of excessive local irritation and/or systemic toxicity.
- Only the highest dose of a substance is tested in the rLLNA (Kimber et al. 2006).

Because the criteria for choosing the highest dose in the traditional LLNA and in the rLLNA are the same, the maximum dose level tested in the traditional LLNA and that tested in the rLLNA should be the same.

ICCVAM (1999) compared the accuracy and reliability of the traditional LLNA to guinea pig skin sensitization tests (EPA 2003) and to human data. ICCVAM concluded that:

- The LLNA was a valid alternative to currently accepted guinea pig test methods for most testing situations.
- The LLNA reduced the number of animals required for testing while also refining the procedure by eliminating animal pain and distress.

The LLNA was subsequently accepted by U.S. regulatory agencies as an alternative to the guinea pig tests for assessing the potential of substances to cause ACD.

Accuracy of the rLLNA

The accuracy of the rLLNA for identifying potential skin sensitizers was compared to that of the traditional LLNA.

In the 471 traditional LLNA studies, 318 results were positive and 153 were negative.

When studies in which substances were tested more than once in the same vehicle were combined[1] to yield an overall skin-sensitization classification, 465 studies with unique combinations of substances and vehicles were evaluated, with 315 classified as sensitizers and 150 classified as nonsensitizers. As indicated in Table 1, six substances were positive in the traditional LLNA based on an SI ≥ 3 at a dose other than the highest dose (i.e., false negative in the rLLNA; see Figure 1). Since the rLLNA only evaluates the highest dose tested, all six substances were incorrectly identified as nonsensitizers when compared to the traditional LLNA.

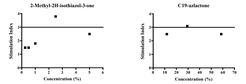
[1]Due to the small number of repeated studies (5% of total studies), all studies were treated independently for the purpose of this accuracy evaluation. When the studies for the substances repeated in the same vehicle were considered together to yield an overall skin sensitization classification, there were 465 studies with unique substance and vehicle combinations.

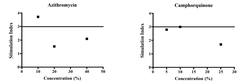
Table 1: Performance of the rLLNA in Predicting Skin Sensitizers Compared to the Traditional LLNA

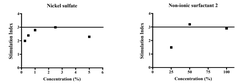
Data	N	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
Kimber et al. (2006)	211	98.6% (208/211)	98.2% (166/169)	100% (42/42)	0% (0/42)	1.8% (3/169)
rLLNA	471	98.7% (465/471)	98.1% (312/318)	100% (153/153)	0% (0/153)	1.9% (6/318)
rLLNA (substances repeated in the same vehicle considered together)	465	98.7% (459/465)	98.1% (309/315)	100% (150/150)	0% (0/150)	1.9% (6/315)

Abbreviations: N = Number of studies; rLLNA = Reduced murine local lymph node assay

Figure 1: Dose-Response Curves for the Six False Negative Substances in the rLLNA







Interlaboratory Reproducibility of the rLLNA

Interlaboratory reproducibility of the rLLNA was assessed with traditional LLNA data for five substances tested independently in the same vehicle at two or three laboratories:

- Dinitrochlorobenzene (DNCB)
- Hexyl cinnamic aldehyde (HCA)
- Linalool alcohol
- Methyl salicylate
- Potassium dichromate

All studies correctly classified DNCB and potassium dichromate as sensitizers and methyl salicylate as a nonsensitizer (i.e., 100% concordance).

HCA and linalool alcohol, which were tested independently in two laboratories, were classified as sensitizers by one traditional LLNA study and as nonsensitizers by the other study. Review of these two studies indicates that the discordant results were due to differences in the highest dose levels tested.

However, because the rLLNA and traditional LLNA use identical protocols and the data sets used to evaluate their accuracy are similar, the intra- and interlaboratory reliability of the rLLNA was deemed to be similar to that of the traditional LLNA.

ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the scientific validity of the rLLNA has been adequately evaluated and that the performance of the rLLNA, when conducted in accordance with the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2008a), is sufficient to distinguish between skin sensitizers and nonsensitizers in cases that do not require dose–response information.

- Compared to the traditional LLNA, the rLLNA will reduce animal use by 40% for each test.
- ICCVAM recommends that the rLLNA should be used routinely to determine the ACD potential of chemicals and products before conducting the traditional LLNA.
  - Negative substances can be classified as nonsensitizers, and positive substances can be classified as sensitizers.
  - In cases that require dose–response information, positive substances must be tested in the traditional multiple-dose LLNA.
  - If dose–response information is required for a substance that is considered likely to produce ACD, it should be evaluated initially using the traditional LLNA rather than the rLLNA.
- There is a small possibility of a false negative result (1.9% [6/318]) in the rLLNA compared to the traditional LLNA.
  - This information should be considered when evaluating results from the rLLNA, and negative results should always prompt an integrated assessment of supplemental information (e.g., possibility of downturn in response at the high dose, test results with similar substances, peptide-binding activity, molecular weight, other testing data).
  - If false negative results are suggested, confirmatory testing in the traditional LLNA or another accepted skin-sensitization test method should be considered.

ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends that the rLLNA should be conducted according to the updated ICCVAM-recommended LLNA test method protocol. Key aspects include:

- The highest concentration used should be the maximum soluble concentration that does not induce excessive local irritation and/or overt systemic toxicity (see protocol in Appendix A of ICCVAM 2008a for procedures).
- Individual animal data should be collected.
  - This will allow for the identification and exclusion of outlier values that could cause false negative or false positive results.
  - Collection of individual animal data (versus pooled) also allows for statistical analysis to determine whether the test-substance response is significantly different from that of the vehicle control.
- A minimum of four animals per dose group should be used.
  - Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429 for the LLNA currently requires at least five animals per dose group if individual data are collected but only four animals in each dose group if lymph nodes from all animals in the group are pooled into one sample for data collection (OECD 2002).
  - Statistical analyses indicate that reducing dose groups from five animals to four is unlikely to significantly affect the results of an LLNA study.
  - This revision is important because many national regulations and policies require that the minimum number of animals be used for studies. Therefore, once TG 429 is updated with the revision, the collection of individual animal data will be consistent with this requirement.
- A positive-control substance should be used with each test.
  - This will determine if the conduct of protocol procedures and all aspects of the test system are responding adequately to produce a positive response.

ICCVAM Recommendations: Future Studies

ICCVAM recommends additional studies to further characterize and potentially improve the usefulness and applicability of the rLLNA for identifying potential skin sensitizers. These include:

- Additional efforts should be made to understand the basis for abnormal dose responses for the six substances in this evaluation that would have resulted in false negative results using the rLLNA compared to the traditional LLNA.
  - This information should help identify ways to improve the accuracy of the rLLNA compared to the traditional LLNA.
  - Efforts should also be made to identify data from guinea pigs and humans for these and other substances that exhibit abnormal dose responses in the traditional LLNA, including information from post-marketing surveillance and/or occupational exposures.
- All future traditional LLNA and rLLNA studies should collect individual animal data.
  - This will allow detection of outliers and avoidance of false negative results that can occur from pooling data that include one or more abnormally low values.
  - Existing LLNA studies using data pooled from all animals in a dose group, such as four of the six false negative rLLNA results in this evaluation, should be evaluated further with data obtained from individual animals within each dose group to determine if pooling of data may have led to false negative outcomes.
- Data from individual animals should be collected and analyzed to identify opportunities to use fewer animals per dose group without compromising test method accuracy.
  - The updated ICCVAM-recommended LLNA test method protocol incorporates statistical procedures necessary for such determinations.
  - The updated protocol also includes guidance for determining if the number of animals in the concurrent positive-control group can be reduced by evaluating the laboratory's historical positive-control database.

ICCVAM Recommendations: Performance Standards

- The ICCVAM-recommended test method performance standards for the traditional LLNA may be used to evaluate the performance of modified test methods, including the rLLNA, that are functionally and mechanistically similar to the traditional LLNA. Modified protocols for the rLLNA that adhere to the traditional LLNA performance standards would be considered acceptable for hazard identification purposes.
- The ICCVAM Performance Standards for the Murine Local Lymph Node Assay: Methods For Assessing Lymphocyte Proliferation are reviewed on Poster 2036, Board 144, Thursday morning in the Regulations and Policy Implications in Toxicology Session (ICCVAM Performance Standards for the Murine Local Lymph Node Assay (LLNA). J. Matheson; M. Wind; A. Jacobs; D. Allen; T. Burns; E. Salicru; J. Strickland; R. Tice; W. Stokes). The document can be accessed at: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm

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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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NTP

ICCVAM-recommended rLLNA Protocol

Days 1 – 3. Apply 25 µL test substance in appropriate vehicle to dorsum of both ears of each of four mice in each treatment (highest dose that would be tested in the traditional LLNA) or control group.

↓

Days 4 – 5. No treatment

↓

Day 6. Inject 20 µCi ^{3}H-methyl thymidine or 2 µCi ^{125}I-iododeoxyuridine and 10^{-5} M fluorodeoxyuridine into the tail vein of each mouse.

↓

After 5 hours, harvest lymph nodes, crush, and prepare a single-cell suspension.

↓

Wash the single-cell suspension twice with phosphate buffered saline and then precipitate the DNA with 5% trichloroacetic acid at 4°C for 18 hours.

↓

Prepare for counting radioactivity by resuspending the pellet in trichloroacetic acid and adding scintillation fluid (for ^{3}H), or by adding resuspended pellets to gamma counting tubes (for ^{125}I).

↓

Count radioactivity. Average dpm for control group and treatment groups. Calculate stimulation index (SI) as:

$$SI = \frac{\text{Treatment group mean dpm}}{\text{Control group mean dpm}}$$

SI ≥ 3 classifies substances as sensitizers.
SI < 3 classifies substances as nonsensitizers.

Timeline for the ICCVAM Evaluation of the Reduced Murine Local Lymph Node Assay

Date	Event
January 10, 2007	ICCVAM receives request from the CPSC nominating six LLNA review activities, including evaluation of the rLLNA.
January 2007	ICCVAM IWG re-established to work with NICEATM to carry out LLNA evaluations.
January 24, 2007	ICCVAM endorses the six 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 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	• Independent Peer Review Panel Meeting, CPSC Headquarters, Bethesda, MD. • Public meeting with opportunity for oral public comments. • Panel reviewed the current validation status of the rLLNA, and commented on extent that information in the draft ICCVAM rLLNA BRD supported the draft ICCVAM test method recommendations.
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.*
June 18-19, 2008	SACATM public meeting for comments on the Panel report.
October 29, 2008	ICCVAM endorses the TMER for the rLLNA test method, which includes the final rLLNA BRD.

Abbreviations: BRD = Background Review Document; CPSC=U.S. Consumer Product Safety Commission; ECVAM=European Centre for the Validation of Alternative Methods; 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; rLLNA=Reduced murine local lymph node assay; SACATM=Scientific Advisory Committee on Alternative Toxicological Methods; TMER=Test Method Evaluation Report

LLNA Peer Review Panel Meeting

A public meeting of an independent scientific peer review panel organized by the ICCVAM and NICEATM was held at the Consumer Product Safety Commission in Bethesda, MD, on March 4-6, 2008.

Charge to the Peer Review Panel Regarding LLNA Performance Standards

- *Review the rLLNA BRD for errors and omissions*
- *Provide conclusions and recommendations on the current validation status of the rLLNA test method.*
- *Does the information contained in the draft BRD supported ICCVAM's draft test method recommendations?*

Independent Scientific Peer Review Panel

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Conclusions

- ICCVAM concludes that the performance of the rLLNA, when conducted in accordance with the updated ICCVAM-recommended LLNA protocol, is sufficient to distinguish between skin sensitizers and nonsensitizers.
- ICCVAM recommends that the rLLNA should be used routinely to determine the ACD potential of chemicals and products before conducting the traditional LLNA.
- Compared to the traditional LLNA, the rLLNA will reduce animal use by 40% for each test.
- There is a small possibility of a false negative result (1.9% [6/318]), if false negative results are suspected confirmatory testing in the traditional LLNA or another accepted skin-sensitization test method should be considered.
- In cases that require dose–response information, positive substances must be tested in the traditional multiple dose LLNA.

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