The Updated ICCVAM Recommended Murine LLNA Test Method Protocol

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Introduction

The United States and international regulatory authorities currently accept the murine local lymph node assay (LLNA) as an alternative to guinea pig test methods for skin sensitization testing. In March 2008, the Interagency Coordinating Committee on the Validation of Alternative Toxicological Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) convened an independent international peer review panel (the "Panel") to evaluate the usefulness and limitations of new versions and applications of the LLNA. The Panel provided several recommendations that were applicable to the LLNA protocol. ICCVAM subsequently finalized an updated LLNA protocol to include:

1. Reducing the required number of animals from five to four per group
2. Scientific rationale for collection of individual animal data
3. Guidance for use of a concurrent positive control group
4. Guidance on evaluating local irritation and systemic toxicity to establish the appropriate highest dose to test

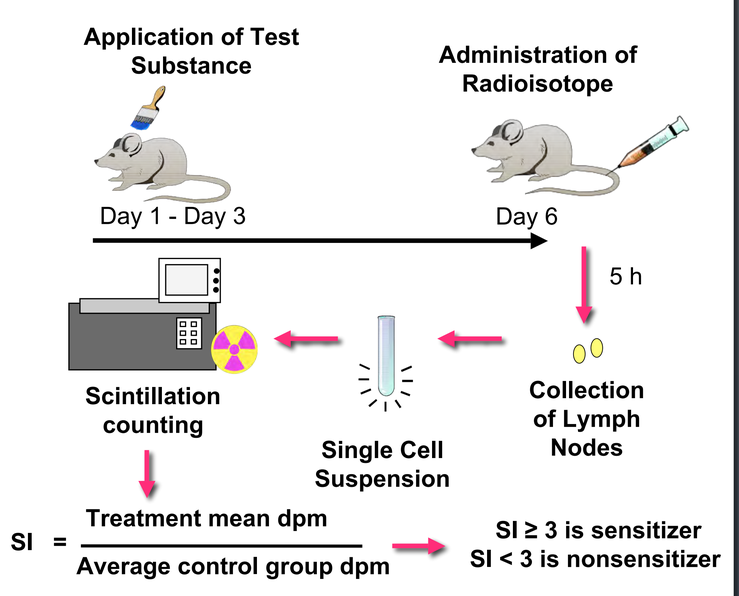
Updated ICCVAM Recommended Test Method Protocol for the LLNA

- U.S. Federal agencies are interested in a standardized protocol for skin sensitization hazard classification.
- NICEATM submitted a proposed update to Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429 (OECD 2002).
- The test method protocol was a key component of performance standards for the LLNA (ICCVAM 2009a).

Basic Principles of the LLNA

- Sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of substance application.
- Proliferation is a function of *in vivo* radioisotope incorporation into the DNA of dividing lymphocytes.
- Proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization.

Methodology of the LLNA



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ECPA (European Crop Protection Association), Belgium
EFfCI (European Federation for Cosmetic Ingredients), Belgium

This poster reflects the views of the authors and has not been reviewed or approved by the U.S. Consumer Product Safety Commission or other agencies.

Reducing from Five to Four Animals per Group

- 83 LLNA studies (275 dose groups) from six labs were evaluated.
  - The average agreement (N = 4 vs. N = 5) was 97.5%.
  - A reduction in the sample size N = 5 to N = 4 is unlikely to have a significant impact (**Table 1**).
- This change is important since most animal-use regulations require that the minimum number of animals be used in studies.
- The current OECD TG 429 specifies four animals/group for pooled data and five animals/group for individual animal data.
  - Only pooled data are collected in many countries because it requires fewer animals.
  - An update to TG 429 is being proposed so that only four animals are required for collection of individual animals.

Table 1. Agreement Between Sample Sizes for LLNA Outcomes of 275 Dose Groups

SI	Frequency of SI	Agreement of Outcomes[1] (%)
< 2.1	154	100.0
2.1 – 2.5	16	90.1
2.6	2	85.0
2.7	3	73.3
2.8	2	59.5
3.1	1	56.0
3.2	2	55.5
3.3	4	73.5
3.4	1	88.0
3.5	1	68.0
3.6	1	84.0
3.7	1	90.0
3.8	1	100.0
4.0 – 4.7	16	97.9
> 4.7	70	100.0
Total	275	97.5

Abbreviation: SI = stimulation index.
[1] Proportion of samples with SI ≥ 3 and SI < 3

Rationale for Collection of Individual Animal Data

- Allows for identification of outlier responses
  - Avoids false negative results for weaker sensitizers
- Allows for assessment of interanimal variability
- Allows for a statistical comparison of the test substance and vehicle control group measurements
- Allows for evaluation of statistical power for different group sizes
- Allows for an evaluation of the impact of reducing the number of animals in the positive control group

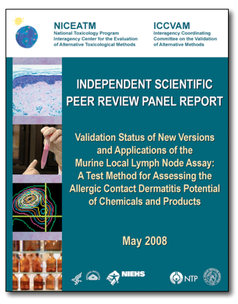
Guidance for Use of a Concurrent Positive Control Group

- A concurrent positive control is specified to ensure that all aspects of the assay are performed in a way that will produce positive results.
- Positive control dose should be reproducible but not excessive.
- There may be certain regulatory situations where it is necessary to test the positive control substance in both a standard and a non-standard vehicle (e.g., a clinically/chemically relevant formulation) to test for possible interactions.

Highlights from the LLNA Peer Review Panel Meeting

Panel Meeting

- A public meeting of an independent scientific peer review Panel organized by ICCVAM and NICEATM was held on March 4-6, 2008.
- The Panel report is available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf



Conclusions Relevant to the LLNA Test Method Protocol

- Data should be collected at the level of individual animals to allow an estimate of the variance within control and treatment groups.
- A concurrent positive control should be run with each test substance to ensure that the system is operating as expected and technical errors are not occurring.
- If a laboratory has extensive historical data indicating that the positive control consistently yields statistically bioequivalent results in the LLNA, then, on a regular periodic basis, evaluation of a positive control could be recommended.

Independent Scientific Peer Review Panel

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Guidance on Relating Local Irritation and Systemic Toxicity with the Highest Dose to Test

- The highest LLNA dose is the maximum soluble concentration that does not induce systemic toxicity or excessive local irritation.
- In the absence of such information, a prescreen test should be performed using three dose levels of the test substance.
  - The prescreen test is conducted the same as the main LLNA study, except for no assessment of lymph node cell proliferation.
  - Six mice (two per concentration) are used.
  - All mice are observed daily for any clinical signs of systemic toxicity or local irritation at the application site.
  - Body weights are recorded pre-test and on Day 6.
  - Both ears of each mouse are observed for erythema and scored using Table 2.
  - Ear thickness measurements are recorded on Day 1 (pre-dose), Day 3 (~48 hours after first dose), and Day 6.
  - Excessive irritation is indicated by an erythema score ≥3 (**Table 2**) or ear swelling of ≥25% (Reeder et al. 2007; ICCVAM 2009b).

Table 2. Erythema Scores

Observation	Value
No visual effect	0
Slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema (beet redness)	3
Eschar (i.e., piece of dead tissue that is cast off from the surface of the skin)	4

- The following clinical observations, which are based on test guidelines and current practices (ICCVAM 2009c), may indicate systemic toxicity when used as part of an integrated assessment and therefore may indicate that the maximum dose recommended for the LLNA has been exceeded:
  - Changes in nervous system function (e.g., piloerection, ataxia, tremors, and convulsions)
  - Changes in behavior (e.g., aggressiveness, change in grooming activity, marked change in activity level)
  - Changes in respiratory patterns (i.e., the frequency and intensity of breathing such as dyspnea, gasping, and rales)
  - Changes in food and water consumption
  - Lethargy and/or unresponsiveness
  - Any signs of more than slight/momentary pain and distress
  - Reduction in body weight >10% from Day 1 to Day 6
  - Mortality

International Acceptance

- A proposal to update OECD TG 429 (the LLNA) has been submitted to include recommendations described herein and is currently under review by the OECD Test Guidelines Program.
  - A minimum of four animals per test group with a collection of individual animal data rather than pooled data
  - Guidance for a prescreen test to select the highest dose for testing based on the maximum soluble concentration and the absence of systemic toxicity and/or excessive local irritation

Conclusions

- ICCVAM-recommended LLNA test method protocol
  - Reduces the minimum number of animals required from 5 to 4 per group
  - Includes rationale for collecting individual animal data and using a concurrent positive control in each LLNA study
  - Provides guidance on the appropriate highest dose to test

References

ICCVAM. 2009a. Recommended Performance Standards: Murine Local Lymph Node Assay. NIH Publication No. 09-7357. http://iccvam.niehs.nih.gov/methods/immunotox/PerfStds/llna-ps.htm

ICCVAM. 2009b. Nonradioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol (LLNA: BrdU-FC) Revised Draft Background Review Document. http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/BRDcomplete.pdf

ICCVAM. 2009c. Report on the ICCVAM-NICEATM/ECVAM/JaCVAM Scientific Workshop on Acute Chemical Safety Testing: Advancing In Vitro Approaches and Humane Endpoints for Systemic Toxicity Evaluations. http://iccvam.niehs.nih.gov/methods/acutetox/Tox_workshop.htm

OECD. 2002. Guideline for the Testing of Chemicals 429 - Skin Sensitisation: Local Lymph Node Assay. Paris: OECD. http://www.oecd.org

Reeder MK, Broomhead YL, DiDonato L, DeGeorge GL. 2007. Use of an enhanced local lymph node assay to correctly classify irritants and false positive substances. Toxicologist 96 (S-1):235.

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