Evaluation of the Murine Local Lymph Node Assay (LLNA) for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans

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Introduction

- The murine local lymph node assay (LLNA) is 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 substance.

- In response to a nomination by the U.S. Consumer Product Safety Commission in 2007, ICCVAM and NICEATM evaluated the LLNA as a stand-alone test method to determine potency categorization of chemicals that may cause ACD in humans.

- The United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) was revised in 2009 to include the option of delineating strong skin sensitizers (Subcategory 1A) from all other skin sensitizers (Subcategory 1B) (UN 2009).
  - Classification criteria for human and LLNA data are based on:
    - Induction concentration in the human repeat-insult patch test (HRIPT) or the human maximization test (HMT) of \( \leq 500 \, \mu g/cm^2 \) for Subcategory 1A and \( > 500 \, \mu g/cm^2 \) for Subcategory 1B
    - LLNA EC3 value (estimated substance concentration that produces a stimulation index of 3) of \( \leq 2\% \) for Subcategory 1A and \( > 2\% \) for Subcategory 1B
LLNA Test Method Protocol

- ICCVAM recommends use of the recently updated LLNA test method protocol (Figure 1) (ICCVAM 2010). The updated LLNA protocol:
  - Includes improved dose selection procedures to guide selection of the highest dose that will help minimize false negatives
  - Provides for a 20% reduction in the required number of animals compared to the previously recommended LLNA protocol (reduces the number of required animals per group from 5 to 4)
  - Recommends collection of individual animal data
  - Recommends inclusion of both a concurrent vehicle control and a positive control in each study
  - Provides procedures for calculating the LLNA EC3, which is necessary for potency determination
Figure 1  LLNA Test Method Protocol

Abbreviations: DPM = disintegrations per minute; SI = stimulation index
Current Validation Status of the LLNA to Classify Strong Human Sensitizers

- A database of 136 substances with LLNA and human data was used for the analysis.
  - LLNA data from positive tests were expressed as EC3 values.
  - Human data from positive HMT or HRIPT were expressed as DSA05 values
    - DSA05 = induction dose per skin area (DSA) that produces a positive response in 5% of the tested population.
  - Both LLNA EC3 and human DSA05 values are thresholds for a positive response.
  - Substances with multiple LLNA EC3 or human DSA05 values were assigned geometric mean values.

- LLNA EC3 ≤ 2% correctly classified 52% (14/27) of the strong human skin sensitizers (Subcategory 1A) (Table 1).
  - 48% (13/27) of strong human skin sensitizers were underclassified as either Subcategory 1B skin sensitizers (11 substances produced LLNA EC3 > 2%) or as nonsensitizers (2 substances).

- The rates of correct and underclassification by the LLNA for the 27 strong human skin sensitizers are shown in Figure 2.
  - As the LLNA EC3 increases, the correct potency classification rate for strong human skin sensitizers increases and the underclassification rate decreases.
− The correct classification rate plateaus, however, because the two strong human skin sensitizers that yielded negative results in the LLNA will not be correctly classified by any EC3 cutoff.

− 14% (11/77) of substances with LLNA EC3 > 2% are strong human skin sensitizers (DSA_{05} \leq 500 \mu g/cm^2).

− 5% (2/38) of the LLNA negative substances were strong human skin sensitizers.
### Table 1. EC3 Values for 27 Strong Human Sensitizers

<table>
<thead>
<tr>
<th>Chemical</th>
<th>EC3 (%)</th>
<th>DSA_{0.05} (µg/cm²)</th>
<th>Chemical</th>
<th>EC3 (%)</th>
<th>DSA_{0.05} (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Chloro)methylisothiazolinone</td>
<td>0.01</td>
<td>5</td>
<td>2-Hexylidene cyclopentanone</td>
<td>2.40</td>
<td>255</td>
</tr>
<tr>
<td>2,4-Dinitrochlorobenzene</td>
<td>0.04</td>
<td>3</td>
<td>Methyl-2-nonynoate</td>
<td>2.50</td>
<td>79</td>
</tr>
<tr>
<td>Tetrachlorosalicylanilide</td>
<td>0.04</td>
<td>27</td>
<td>Diethylmaleate</td>
<td>3.27</td>
<td>400</td>
</tr>
<tr>
<td>4-Phenylendiamine</td>
<td>0.12</td>
<td>30</td>
<td>Diethylenetriamine</td>
<td>3.30</td>
<td>411</td>
</tr>
<tr>
<td>Potassium dichromate</td>
<td>0.12</td>
<td>106</td>
<td>delta-Damascone</td>
<td>3.51</td>
<td>193</td>
</tr>
<tr>
<td>Mercuric (II) chloride</td>
<td>0.39</td>
<td>225</td>
<td>Benzyldiene acetone</td>
<td>3.70</td>
<td>299</td>
</tr>
<tr>
<td>Gold chloride</td>
<td>0.48</td>
<td>98</td>
<td>trans-2-Hexenal</td>
<td>3.78</td>
<td>49</td>
</tr>
<tr>
<td>Methyl-2-octynoate</td>
<td>0.50</td>
<td>388</td>
<td>Phenylacetaldehyde</td>
<td>4.99</td>
<td>329</td>
</tr>
<tr>
<td>Cobalt (II) salts</td>
<td>0.57</td>
<td>279</td>
<td>Benzoisothiazolione</td>
<td>7.79</td>
<td>50</td>
</tr>
<tr>
<td>Beryllium sulfate</td>
<td>0.68</td>
<td>11</td>
<td>Methylisalicylidene acetone</td>
<td>9.30</td>
<td>412</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>0.75</td>
<td>345</td>
<td>Butyl glycidyl ether</td>
<td>30.90</td>
<td>437</td>
</tr>
<tr>
<td>Methylisothiazolinone</td>
<td>0.87</td>
<td>224</td>
<td>Nickel (II) salts</td>
<td>Neg</td>
<td>27</td>
</tr>
<tr>
<td>Cinnamic aldehyde</td>
<td>1.00</td>
<td>382</td>
<td>Streptomycin</td>
<td>Neg</td>
<td>245</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1.40</td>
<td>191</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: DSA_{0.05} = induction dose per skin area in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the test population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, the threshold for a substance to be considered a sensitizer in the LLNA; Neg = negative.

In order of increasing EC3. Some EC3 and DSA_{0.05} values are geometric means of multiple values.
Analysis was based on 27 substances identified as strong skin 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 $\leq 500 \mu g/cm^2$. 

Figure 2  LLNA EC3 Classification of 27 Strong Human Skin Sensitizers
Test Method Usefulness and Limitations

- ICCVAM concludes that the LLNA, using the GHS classification criteria, can be used to categorize substances as Subcategory 1A when the EC3 ≤ 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 substance as Subcategory 1B when the substance produces an LLNA EC3 > 2%.

Future Studies

- In order to develop a more accurate assessment of strong human skin sensitizers using LLNA results, especially for substances that produce EC3 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

  - Existing data from similar chemical entities
An international independent scientific peer review panel considered the NICEATM-ICCVAM evaluation in a public meeting at the U.S. Consumer Product Safety Commission in Bethesda, MD, on March 4-6, 2008.

**Charge to the Peer Review Panel**

- Review the draft Background Review Document (BRD) for errors and omissions
- Provide conclusions and recommendations on the current validation status of the LLNA as a test method to determine potency category
- Does the information contained in the draft BRD support ICCVAM’s draft test method recommendations?

**Peer Review Panel Conclusions**

- Agreed with the ICCVAM draft recommendation that the LLNA should not be considered as a stand-alone test method for determining skin sensitization potency but could be used as part of a weight-of-evidence evaluation.
- Suggested that additional analyses, which are reflected in this poster, might improve the correlation between the LLNA EC3 values and the human threshold values

- Concurred with ICCVAM’s recommendations for future studies

- The complete LLNA Peer Review Panel Report can be accessed at:
Independent Scientific Peer Review Panel

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References

ICCVAM. 2010. 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. Available at: 


Acknowledgments

The Intramural Research Program of the National Institute of Environmental Health Sciences (NIEHS) supported this poster. Technical support was provided by ILS, Inc., under NIEHS contract N01-ES 35504.

This poster reflects the views of the authors. The views expressed above have not been reviewed or approved by the U.S. Consumer Product Safety Commission or any other U.S. Federal agency and do not necessarily represent the official positions of any Federal agency.

Since the poster was written as part of the official duties of the authors, it can be freely copied. NICEATM and ICCVAM gratefully acknowledge the following individuals and institutions that submitted data to NICEATM that were used for this evaluation.

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