The Reduced Murine Local Lymph Node Assay (rLLNA) – Validation Status and Appropriate Use

David Allen, Ph.D.
ILS Inc., Contractor Supporting NICEATM


January 20, 2011
William H. Natcher Conference Center
National Institutes of Health
Bethesda, MD
Overview of the rLLNA - 1

- Modification of multi-dose murine local lymph node assay (LLNA) that uses fewer animals to assess the allergic contact dermatitis (ACD) hazard potential of chemicals and products
  - For each test substance, rLLNA tests only the highest dose vs. at least three doses for LLNA
  - rLLNA reduces animal number by 40% for each test vs. multi-dose LLNA

Abbreviations: GPMT/BT = guinea pig maximization test/Buehler test
Overview of rLLNA - 2

- The rLLNA (radioactive and nonradioactive LLNA) should be used for most testing since 80% of chemical products are nonsensitizers in standardized tests\textsuperscript{1}

LLNA Test Method Protocol

Apply 25 µl Test Substance → No Treatment → Administer Radioisotope ($^{3}$H or $^{125}$I)

Days 1 - 3 → Days 4 - 5 → Day 6

Apply 25 µl Test Substance → No Treatment → Administer Radioisotope

Abbreviations: DPM = disintegrations per minute; SI = stimulation index

Measure Proliferation (Scintillation Counts) → Prepare Single Cell Suspension → Collect Draining Auricular Lymph Nodes

$SI = \frac{\text{Mean DPM of Treatment Group}}{\text{Mean DPM of Vehicle Control Group}}$

SI $\geq$ 3 = Sensitizer (Positive)
SI < 3 = Nonsensitizer (Negative)

Abbreviations: DPM = disintegrations per minute; SI = stimulation index
rLLNA Test Method Protocol - 1

Apply 25 µl Test Substance: (Only Highest Dose)

Days 1 - 3

No Treatment

Days 4 - 5

Administer Radioisotope ($^3$H or $^{125}$I)

Day 6

Day 6 (5 hours)

Measure Proliferation (Scintillation Counts)

Prepare Single Cell Suspension

Collect Draining Auricular Lymph Nodes

SI = \frac{\text{Mean DPM of Treatment Group}}{\text{Mean DPM of Vehicle Control Group}}

SI \geq 3 = \text{Sensitizer (Positive)}
SI < 3 = \text{Nonsensitizer (Negative)}

Abbreviations: DPM = disintegrations per minute; SI = stimulation index
rLLNA Test Method Protocol - 2

- Criteria for selecting highest dose is the same as for multi-dose LLNA:
  - Maximum concentration that does not induce overt systemic toxicity and/or excessive local skin irritation

- Identify existing information to aid in selecting the appropriate maximum dose
  - Acute toxicity data
  - Dermal irritation data
  - Dose data from LLNA tests for structurally related substance(s)

- In absence of existing information, a prescreen test may be necessary
  - Identical experimental conditions except for:
    - Omission of lymph node cell proliferation assessment
    - Fewer animals per dose group
Use of a reduced procedure by testing only the highest dose is applicable to the radioactive LLNA and the nonradioactive LLNA

- Radioactive rLLNA (stimulation index [SI] ≥ 3.0 decision criterion)
- Nonradioactive reduced LLNA: BrdU-ELISA (SI ≥ 1.6 decision criterion)
- Nonradioactive reduced LLNA: DA (SI ≥ 1.8 decision criterion)
Minimum of four animals per group

Individual animal data
- Allows for statistical analysis for detection of outliers and comparison to vehicle control group

Concurrent vehicle control
- Used as the baseline to determine any increase in lymphocyte proliferation of treated animals

Concurrent positive control
- Demonstrates that the assay as conducted is capable of producing a positive response
- Required by U.S. agencies
  - Absence of a concurrent positive control could result in a requirement to repeat negative results
NICEATM-ICCVAM Evaluation of rLLNA

- Reviewed available data and information regarding the usefulness and limitations to assess the ACD hazard potential of chemicals and products

- Determined validation status
  - Accuracy: sensitivity and specificity
  - Reproducibility for identifying LLNA sensitizers and nonsensitizers
  - Scope of substances tested
  - Availability of a standardized test method protocol

- Independent international scientific peer review panel
471 traditional LLNA studies
- Published reports and unpublished data in response to May 17, 2007 Federal Register (FR)¹
- 318 sensitizers (SI ≥ 3)
- 153 nonsensitizers (SI < 3)
- Studies for substances tested more than once in the same vehicle were combined to yield an overall skin-sensitization classification
  - 465 studies with unique substance/vehicle combinations²
  - 315 sensitizers (SI ≥ 3)
  - 150 nonsensitizers (SI < 3)
  - 211 substances in the original ICCVAM LLNA evaluation³

² 457 unique substances but some substances tested in more than 1 vehicle; each substance-vehicle combination considered separately (n = 465).
## NICEATM-ICCVAM Evaluation of rLLNA – Validation Database - 2

<table>
<thead>
<tr>
<th>Data Source</th>
<th>Number of Studies</th>
<th>Primary Data Source and Substance Selection Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerberick et al. (2005)</td>
<td>210</td>
<td>Historical data: Substances of varying skin sensitization potential</td>
</tr>
<tr>
<td>M.J. Olson/GlaxoSmithKline</td>
<td>124</td>
<td>Pharmaceuticals, pharmaceutical intermediates</td>
</tr>
<tr>
<td>Basketter, Gerberick, and Kimber</td>
<td>31</td>
<td>Historical data: Substances of varying skin sensitization potential</td>
</tr>
<tr>
<td>K. Skirda/CESIO (TNO Report V7217)</td>
<td>18</td>
<td>CESIO data in paper: “Limitations of the LLNA as preferred test for skin sensitisation: concerns about false positive and false negative test results”</td>
</tr>
<tr>
<td>Lalko and Api (2006)</td>
<td>17</td>
<td>Essential oils, commonly used in perfumery (contain significant known skin sensitizers)</td>
</tr>
<tr>
<td>H.W. Vohr/BGIA</td>
<td>16</td>
<td>Epoxy resin components</td>
</tr>
<tr>
<td>Ryan et al. (2002)</td>
<td>15</td>
<td>Water-soluble haptens and known skin sensitizers to assess usefulness of a novel vehicle</td>
</tr>
<tr>
<td>D. Germolec/NIEHS</td>
<td>15</td>
<td>Substances evaluated by the National Toxicology Program</td>
</tr>
<tr>
<td>E. Debruyne/Bayer CropScience SA</td>
<td>10</td>
<td>Pesticide types and formulations</td>
</tr>
<tr>
<td>P. Ungeheur/EFfCI</td>
<td>9</td>
<td>Unsaturated chemicals</td>
</tr>
<tr>
<td>P. Botham/ECPA</td>
<td>6</td>
<td>Pesticides evaluated in the LLNA with a novel vehicle</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>471</strong></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BGIA = German Institute for Occupational Safety and Health; CESIO = European Committee on Organic Surfactants and their Intermediates; ECPA = European Crop Protection Association; EFfCI = European Federation for Cosmetic Ingredients; NIEHS = National Institute of Environmental Health Sciences; TNO = Netherlands Organisation for Applied Research.

1 The total number of studies does not take into account the fact that some substances were tested more than once.
### NICEATM-ICCVAM Evaluation of rLLNA – Validation Database - 3

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohols</td>
<td>9</td>
<td>4</td>
<td>Hydrocarbons, Acyclic</td>
<td>2</td>
<td>1</td>
<td>Nitriles</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>21</td>
<td>4</td>
<td>Hydrocarbons, Cyclic</td>
<td>14</td>
<td>7</td>
<td>Nitro Compounds</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Amides</td>
<td>4</td>
<td>0</td>
<td>Hydrocarbons, Halogenated</td>
<td>27</td>
<td>1</td>
<td>Nitroso Compounds</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Amidines</td>
<td>1</td>
<td>0</td>
<td>Hydrocarbons, Other</td>
<td>7</td>
<td>8</td>
<td>Onium Compounds</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Amines</td>
<td>14</td>
<td>7</td>
<td>Imines</td>
<td>0</td>
<td>1</td>
<td>Pharmaceutical chemicals</td>
<td>0</td>
<td>125</td>
</tr>
<tr>
<td>Anhydrides</td>
<td>1</td>
<td>0</td>
<td>Inorganic Chemicals</td>
<td>0</td>
<td>2</td>
<td>Phenols</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>3</td>
<td>2</td>
<td>Isocyanates</td>
<td>1</td>
<td>0</td>
<td>Polycyclic Compounds</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Carboxylic Acids</td>
<td>29</td>
<td>15</td>
<td>Ketones</td>
<td>5</td>
<td>0</td>
<td>Quinones</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Esters</td>
<td>3</td>
<td>0</td>
<td>Lactones</td>
<td>2</td>
<td>2</td>
<td>Sulfur Compounds</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Ethers</td>
<td>14</td>
<td>2</td>
<td>Lipids</td>
<td>7</td>
<td>14</td>
<td>Urea</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Formulations</td>
<td>0</td>
<td>10</td>
<td>Inorganic Chemicals</td>
<td>0</td>
<td>2</td>
<td>Unknown</td>
<td>28</td>
<td>42</td>
</tr>
<tr>
<td>Heterocyclic Compounds</td>
<td>18</td>
<td>4</td>
<td>Macromolecular Substances</td>
<td>0</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
NICEATM-ICCVAM Evaluation of rLLNA – Test Method Accuracy - 1

<table>
<thead>
<tr>
<th>Data</th>
<th>N</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>False Positive Rate</th>
<th>False Negative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kimber et al. 2006</td>
<td>211</td>
<td>98.6%</td>
<td>98.2%</td>
<td>100%</td>
<td>0%</td>
<td>1.8%</td>
</tr>
<tr>
<td>(208/211)</td>
<td></td>
<td>(166/169)</td>
<td>(42/42)</td>
<td></td>
<td>(0/42)</td>
<td>(3/169)</td>
</tr>
<tr>
<td>rLLNA</td>
<td>471</td>
<td>98.7%</td>
<td>98.1%</td>
<td>100%</td>
<td>0%</td>
<td>1.9%</td>
</tr>
<tr>
<td>rLLNA (substances repeated in same vehicle considered together)</td>
<td>465</td>
<td>98.7%</td>
<td>98.1%</td>
<td>100%</td>
<td>0%</td>
<td>1.9%</td>
</tr>
<tr>
<td>(459/465)</td>
<td></td>
<td>(309/315)</td>
<td>(150/150)</td>
<td></td>
<td>(0/150)</td>
<td>(6/315)</td>
</tr>
</tbody>
</table>

N = number of studies
Six substances were positive in the LLNA (SI ≥ 3) at a dose other than the highest dose.

Since the rLLNA only evaluates the highest dose, all six substances were incorrectly identified as nonsensitizers.
### Summary of available physicochemical properties for six false negatives

<table>
<thead>
<tr>
<th>Substance</th>
<th>CASRN</th>
<th>Vehicle</th>
<th>Molecular Weight</th>
<th>$K_{ow}^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Methyl-2H-isothiazol-3-one</td>
<td>2682-20-4</td>
<td>Acetone: olive oil</td>
<td>115.15</td>
<td>0.68</td>
</tr>
<tr>
<td>Nickel sulfate</td>
<td>7786-81-4</td>
<td>Pluronic L92 (1%)</td>
<td>154.76</td>
<td>-0.17</td>
</tr>
<tr>
<td>Camphorquinone</td>
<td>465-29-2</td>
<td>Acetone: olive oil</td>
<td>166.22</td>
<td>2.15</td>
</tr>
<tr>
<td>C19-azlactone</td>
<td>--</td>
<td>Acetone: olive oil</td>
<td>379.63</td>
<td>5.21</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>83905-01-5</td>
<td>Acetone</td>
<td>748.99</td>
<td>3.24</td>
</tr>
<tr>
<td>Non-ionic surfactant 2</td>
<td>--</td>
<td>Acetone: olive oil</td>
<td>---</td>
<td>--</td>
</tr>
</tbody>
</table>

Abbreviations: CASRN = Chemical Abstracts Service Registry Number.

$^1$ $K_{ow}$ represents the octanol-water partition coefficient (expressed on log scale).
NICEATM-ICCVAM Evaluation of rLLNA – Test Method Reproducibility

- Since the rLLNA and multi-dose LLNA use identical protocols and similar data sets, the intra- and inter-laboratory reliability of the rLLNA was deemed similar to that of the multi-dose LLNA

- 1999 ICCVAM evaluation of multi-dose LLNA reproducibility\(^1\), \(^2\):
  - 2,4-dinitrochlorobenzene tested twice in each of five laboratories
  - Hexyl cinnamic aldehyde tested six times in each of two laboratories
  - Analyses indicated a lack of significant intra- and inter-laboratory variability
  - LLNA repeatability and reproducibility was considered acceptable

- Additional data in 2008 (n = 5 chemicals)
  - Analyses consistent with 1999 ICCVAM reproducibility evaluation for the multi-dose LLNA


ICCVAM Test Method Recommendations for rLLNA – Usefulness and Limitations

**Usefulness**

- Sufficient to distinguish between skin sensitizers and nonsensitizers
- Should be used routinely to determine the ACD hazard potential of chemicals and products
  - If existing information suggests a substance might have ACD hazard potential AND dose-response information is needed, consider testing in the multi-dose LLNA

**Limitations**

- Does not provide dose-response information
  - EC3 cannot be calculated
- Small possibility of false negatives (1.9% [6/318]) compared to LLNA validation database
  - When rLLNA conducted for suspected positives, and a negative result is obtained confirmatory testing in the multi-dose LLNA might be considered
Criteria for Deciding to Use the rLLNA

- Use rLLNA routinely to determine the ACD hazard potential of chemicals and products unless there is a likelihood that it is a sensitizer and dose response information is needed.

- Available information and data about the chemical/product to consider include:
  - Physicochemical properties
  - Structural relationship to known skin sensitizers
    - Structural alerts/QSAR
  - *In vitro*/*in silico*/*in chemico* data
  - Human data
  - Test results for similar substances
  - Toxicogenomic data
Decision Strategy for Using rLLNA

Consider all available information, including \textit{in vitro/ in silico/ in chemico} data

ACD Potential? Need for Dose Response Data?

NO for either
- rLLNA initially
  - 12 mice/substance (40\% fewer mice)

YES for both
- Multi-dose LLNA
  - 20 mice/substance

Positive: Classify as sensitizer
Negative: Classify as nonsensitizer
rLLNA International Acceptance

- rLLNA approach provided as an option in updated OECD TG 429 Skin Sensitization: Local Lymph Node Assay
  - Adopted July 22, 2010
  - Available at http://www.oecd-ilibrary.org/environment/test-no-429-skin-sensitisation_9789264071100-en
  - Based on ICCVAM-recommended LLNA protocol
  - Expected to further reduce animal use for ACD assessments on a global basis, while ensuring human safety
See poster at this workshop (Room C1/C2):

**ICCVAM Evaluation and International Acceptance of the Reduced LLNA: an Alternative Test Method Using Fewer Animals to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products**

M Wind¹, J Matheson¹, A Jacobs², D Allen³, T Burns³, J Strickland³, W Stokes⁴

¹U.S. CPSC, Bethesda, MD; ²U.S. FDA, Silver Spring, MD; ³ILS, Inc., Contractor Supporting NICEATM, RTP, NC; ⁴NICEATM/NTP/NIEHS/NIH/DHHS, RTP, NC
### Acknowledgements

ICCVAM and NICEATM gratefully acknowledge the following individuals and institutions for submitting data to NICEATM for the rLLNA evaluation:

<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>David Basketter, Ph.D.</td>
<td>Unilever Safety and Environmental Assurance Centre</td>
<td>Sharnbrook, U.K.</td>
</tr>
<tr>
<td>Phil Botham, Ph.D.</td>
<td>European Crop Protection Association</td>
<td>Brussels, Belgium</td>
</tr>
<tr>
<td>Eric Debruyne, Ph.D.</td>
<td>Bayer CropScience SA, Sophia Antipolis Cedex, France</td>
<td></td>
</tr>
<tr>
<td>G. Frank Gerberick, Ph.D.</td>
<td>The Procter &amp; Gamble Company</td>
<td>Cincinnati, OH</td>
</tr>
<tr>
<td>Dori Germolec, Ph.D.</td>
<td>National Toxicology Program</td>
<td>Research Triangle Park, NC</td>
</tr>
<tr>
<td>Ian Kimber, Ph.D.</td>
<td>Syngenta Central Toxicology Laboratory</td>
<td>Macclesfield, U.K.</td>
</tr>
<tr>
<td>Michael J. Olson, Ph.D.</td>
<td>GlaxoSmithKline</td>
<td>Research Triangle Park, NC</td>
</tr>
<tr>
<td>Kirill Skirda, Ph.D.</td>
<td>TNO Quality of Life</td>
<td>Delft, Netherlands</td>
</tr>
<tr>
<td>Peter Ungeheuer, Ph.D.</td>
<td>European Federation for Cosmetic Ingredients</td>
<td>Frankfurt, Germany</td>
</tr>
<tr>
<td>Hans Werner Vohr, Ph.D.</td>
<td>Bayer HealthCare</td>
<td>Wuppertal-Elberfeld, Germany</td>
</tr>
</tbody>
</table>
Additional Acknowledgements

- ICCVAM
- ICCVAM Interagency Immunotoxicity Working Group
- ICCVAM Independent Scientific Peer Review Panel
- NICEATM Staff