Performance Characteristics of the Local Lymph Node Assay (LLNA) Limit Dose Procedure

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Abstract

ICCVAM recommended the murine LLNA as a valid substitute for guinea pig tests for assessing allergic contact dermatitis in 1999. In 2007, the CPSC requested that NICEATM and ICCVAM evaluate the validation status of the LLNA limit dose approach, a modification proposed by Kimber et al. (2006). In the limit dose procedure, only the high dose is tested compared to testing three or more doses in the standard LLNA. This modification reduces the number of mice used per study by 40% or more. Based on the Kimber et al. retrospective evaluation of LLNA data for 211 chemicals, the LLNA limit dose approach, compared to the LLNA, had an accuracy of 98.6% (208/211), a false positive rate of 0% (0/42), and a false negative rate of 1.8% (3/169). Based on this publication, the ECVAM Scientific Advisory Committee (ESAC) concluded in April 2007 that the LLNA limit dose approach could be used to further reduce the number of animals used for skin sensitization testing. NICEATM subsequently obtained LLNA data for an additional 255 chemicals and formulations that were used to further evaluate the performance characteristics of the LLNA limit dose approach. Compared to the standard LLNA, the LLNA limit dose approach had an accuracy of 98.9% (461/466), a false positive rate of 0% (0/153), and a false negative rate of 1.6% (5/313). Similar to the three false negatives in Kimber et al., the 2 additional false negatives were classified as sensitizers in the standard LLNA based on the low- or middle dose producing an SI≥3, with the highest dose producing an SI<3. This evaluation of an expanded and more diverse group of chemicals supports the proposed use of the LLNA limit dose procedure. ILS staff supported by NIEHS contract N01-ES 35504.

Introduction

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), recommended the murine local lymph node assay (LLNA) as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis (ACD) potential of many, but not all types of substances.

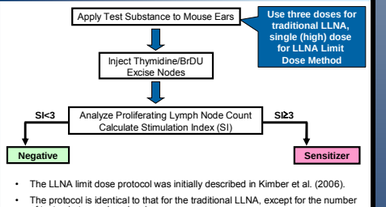
- This recommendation was based on a comprehensive evaluation of data for 211 substances and included an independent scientific peer review panel assessment of the validation status of the LLNA (ICCVAM, 1999a).
- ICCVAM forwarded to U.S. Federal agencies recommendations that the LLNA be considered for regulatory acceptance or other non-regulatory applications for assessing the ACD potential of substances, recognizing that some testing situations would still require the use of traditional guinea pig test methods (ICCVAM 1999a, Sailstad et al. 2001).
- The Panel report and the ICCVAM recommendations (ICCVAM 1999a) are available at the NICEATM/ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf).

The LLNA was subsequently incorporated into the following national and international test guidelines for the assessment of skin sensitization:

- Organisation for Economic Co-operation and Development Test Guideline 429, Skin Sensitisation: Local Lymph Node Assay (OECD 2002)
- International Standards Organization 10993-10: Tests for Irritation and Sensitization (ISO 2002)
- U.S. Environmental Protection Agency Health Effect Testing Guidelines on Skin Sensitization (EPA 2003)

In January 2007, the U.S. Consumer Product Safety Commission formally nominated the LLNA limit dose procedure to ICCVAM for assessment of its scientific validity for regulatory testing applications. (The nomination is available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf)

The LLNA Test Method



- The LLNA limit dose protocol was initially described in Kimber et al. (2006).
- The protocol is identical to that for the traditional LLNA, except for the number of test substance dose levels.
- The traditional LLNA protocol used for the studies evaluated here was consistent with the ICCVAM recommended protocol (ICCVAM 1999, Dean et al. 2001), the EPA test guideline (EPA 2003), or OECD TG 429 (OECD 2002).
- The traditional LLNA uses three dose levels. The highest concentration is that which does not induce systemic toxicity and/or excessive skin irritation.
- The LLNA limit dose procedure uses a single, high dose that does not induce systemic toxicity and/or excessive skin irritation.
- The threshold for classifying a substance as a skin sensitizer is a Stimulation Index (SI) ≥ 3.

Table 1

Summary of Data Sources and Rationale for Substance Selection

Data Source[1]	Number of Studies	Primary Data Source and Substance Selection Rationale
Gerberick et al. (2005)[2]	210	Compiled from previously conducted studies (from published literature and unpublished sources) on substances of varying skin sensitization potential
M.J. Olson/ GlaxoSmithKline	124	Pharmaceuticals, pharmaceutical intermediates
Basketter, Gerberick, and Kimber[3]	31	Compiled from previously conducted studies (from published literature and unpublished sources) on substances of varying skin sensitization potential
K. Skirda/CESIO (TNO Report V7217)	18	Data were provided by CESIO member companies for use in paper titled "Limitations of the Local Lymph Node Assay (LLNA) as preferred test for skin sensitisation: concerns about false positive and false negative test result"
Lalko and Api (2006)	17	Original research conducted on essential oils which were representative of the oils commonly used in perfumery. Each contains significant amounts of one or more known skin sensitizers.
H.W. Vohr/BGIA	16	Original research with epoxy resin components as part of a validation effort for non-radioactive versions of the Local Lymph Node Assay
Ryan et al. (2002)	15	Original research with known water-soluble haptens and known skin sensitizers to assess the usefulness of a novel vehicle
D. Germolec/NIEHS	15	Substances evaluated by the National Toxicology Program for skin sensitization potential
E. Debruyne/Bayer CropScience SA	10	Original research on different pesticide types and formulations
P. Ungeheur/EFfCI	9	Data for selected unsaturated chemicals were provided in the report entitled "Comparative Experimental Study on the Skin Sensitising Potential of Selected Unsaturated Chemicals as Assessed by the Murine Local Lymph Node Assay (LLNA) and the Guinea Pig Maximisation Test (GPMT)"
P. Botham/ECPA	6	Plant protection products (i.e., pesticides) were evaluated in the Local Lymph Node Assay with a novel vehicle to assess its usefulness
Total	**471[4]**	

Abbreviations: BGIA: Berufsgenossenschaftliches Institut fur Arbeitsschutz; CESIO = Comite European des Agents de Surface et de Leurs Intermediaires Organiques; ECPA = European Crop Protection Association; EFfCI = European Federation for Cosmetic Ingredients; NIEHS = National Institute for Environmental Health Sciences; TNO = TNO Nutrition and Food Research

[1]Studies were originally provided for review of the traditional LLNA in 1998, identified from the peer-reviewed literature, or from data submitted to the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in response to a 2007 *Federal Register (FR)* notice. (*FR* notice available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_67_9544.pdf).

[2]These data were evaluated by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee in its evaluation of the LLNA limit dose procedure and were previously submitted to ICCVAM in 1998 for the original evaluation of the validation status of the LLNA (ICCVAM 1999, Gerberick et al. 2005).

[3]Data were included in a submission to ECVAM for the validation of traditional LLNA as a stand-alone assay for potency determination.

[4]The total number of studies does not take into account the fact that some substances were tested more than once. Data from 466 unique substances were reviewed.

Table 2

Chemical Classes[1,2] Represented in the Database

Chemical Class	Number of Substances Original[3]	Number of Substances Additional[3]	Chemical Class	Number of Substances Original[3]	Number of Substances Additional[3]
Alcohols	9	4	Inorganic Chemicals	0	2
Aldehydes	21	4	Isocyanates	1	0
Amides	4	0	Ketones	5	0
Amidines	1	0	Lactones	2	2
Amines	14	7	Lipids	7	14
Anhydrides	1	0	Macromolecular Substances[4]	0	5
Carbohydrates	3	2	Nitriles	1	1
Carboxylic Acids	29	15	Nitro Compounds	2	0
Esters	3	0	Nitroso Compounds	3	0
Ethers	14	2	Onium Compounds	1	0
Formulations[5]	0	10	Pharmaceutical chemicals[6]	0	125
Heterocyclic Compounds	18	4	Phenols	18	2
Hydrocarbons, Acyclic	2	1	Polycyclic Compounds	5	3
Hydrocarbons, Cyclic	14	7	Quinones	1	1
Hydrocarbons, Halogenated	27	1	Sulfur Compounds	20	2
Hydrocarbons, Other	7	8	Urea	3	0
Imines	0	1	Unknown	28	42

[1]Total number of chemical classes does not equal the total number of substances evaluated because some substances were assigned to more than one class and some substances were not assigned to a specific chemical class.

[2]Chemical classes were retrieved from the National Library of Medicine's ChemID Plus database, or assigned using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings classification system (available at http://www.nlm.nih.gov/mesh/meshhome.html).

[3]Total Number of Substances – Original represents the substances evaluated in Kimber et al. (2006). Total Number of Substances – Additional represents the substances received in response to the released *FR* notice (Vol. 72, No. 95, pp. 27815-27817, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_67_9544.pdf).

[4]No chemical class could be assigned, but formulation or macromolecular substance used to identify such common substances.

[5]The chemical classification of pharmaceutical chemicals for the GlaxoSmithKline (GSK) substances, suggested by Dr. Michael Olson of GSK, captures three types of pharmaceutical substances (actives, intermediates, and starting materials).

Results and Discussion

Table 3

Performance Characteristics of the LLNA Limit Dose Procedure in Predicting Skin Sensitizers Compared to the Traditional LLNA

Data	N	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive		False Negative	
		%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Kimber et al. (2006)	211	98.6	208/211	98.2	166/169	100	42/42	100	166/166	93.3	42/45	0	0/42	1.8	3/169
ICCVAM (2008)	471	98.9	466/471	98.4	312/317	100	154/154	100	312/312	96.9	154/159	0	0/154	1.6	5/317
ICCVAM (2008) Substances tested multiple times in the same vehicle combined	466	98.9	461/466	98.4	308/313	100	153/153	100	308/308	96.8	153/158	0	0/153	1.6	5/313

Abbreviations: conc. = concentration; N=Number of tests; No. = Numbers used to calculate percentage.

Table 4

LLNA Data for Five Substances Incorrectly Identified as Negative by the LLNA Limit Dose Procedure

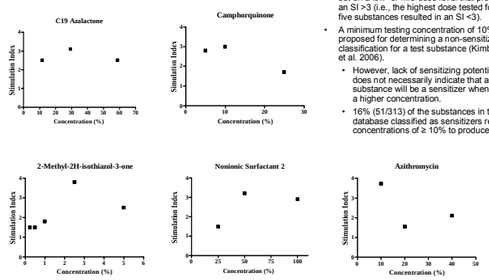
Chemical	EC3	LLNA Data (Low- to Mid-Dose Group)		LLNA Data (Highest Dose Group)	
		Concentration (%)	SI	Concentration (%)	SI
C19-azlactone	26	29.33	3.1	58.67	2.5
Camphorquinone	10	10	3.0	25	1.7
2-Methyl-2H-isothiazol-3-one	1.9	2.5	3.8	5.0	2.5
Azithromycin	NC[1]	10	3.72	40	2.1
Non-ionic surfactant 2	47.1	50	3.2	100	2.9

Abbreviation: NC = Not Calculated; SI = Stimulation Index.

[1] Not calculated because a concentration that produced an SI less than 3 was not evaluated. Therefore, interpolation between points that bracket an SI of 3 was not possible.

Figure 1

Dose-Response Graphs for False Negatives, as Identified by the LLNA Limit Dose Procedure



- The traditional LLNA classification of the five false negative substances as skin sensitizers was not based on the highest tested dose, but on a low- or mid-dose level that produced an SI >3 (i.e., the highest dose tested for these five substances resulted in an SI <3).
- A minimum testing concentration of 10% was proposed for determining a non-sensitizing classification for a test substance (Kimber et al. 2006).
  - However, lack of sensitizing potential at 10% does not necessarily indicate that a substance will be a sensitizer when tested at a higher concentration.
  - 16% (51/313) of the substances in the current database classified as sensitizers required concentrations of ≥ 10% to produce an SI ≥ 3.

Table 5

Summary of Available Physicochemical Properties for False Negatives, as Identified by the LLNA Limit Dose Procedure

Chemical	CASRN	Vehicle	Molecular Weight (g/mol)	K_{OW}[1]	Peptide Reactivity
C19-azlactone	--	Acetone:Olive Oil	379.63	5.21[2]	--
Camphorquinone	465-29-2	Acetone:Olive Oil	166.217	2.15[2]	--
2-Methyl-2H-isothiazol-3-one	2682-20-4	Acetone:Olive Oil	115.15	0.68[2]	High[3]
Azithromycin	83905-01-5	Acetone	748.985	3.243[4]	--
Non-ionic surfactant 2	--	Acetone:Olive Oil	--	--	--

Abbreviations: CASRN = Chemical Abstracts Service Registry Number.

[1] K_{OW} represents the octanol-water partition coefficient (expressed on log scale).

[2] K_{OW} calculated by the method of Moriguchi et al. (1994) and provided in Gerberick et al. (2006).

[3] See Gerberick et al. (2007) for specific peptide reactivity data for this substance.

[4] K_{OW} calculated by the method of Meylan and Howard (1995) and obtained from the website: http://www.syrres.com/esc/est_kowdemo.htm.

- No consistent patterns for these five substances with regard to physicochemical properties were observed.
- No peptide binding activity was available for four of the five substances.

Table 6

LLNA Limit Dose Procedure Responses for Repeated Studies

Chemical	Data Source	Vehicle	LLNA Limit Dose Procedure Response					LLNA Limit Dose Procedure Classification[2]
			Conc (%) /SI	Conc (%) /SI	Conc (%) /SI	Conc (%) /SI	Conc (%) /SI	
Hexyl cinnamic aldehyde	Data Submitted by H.W. Vohr	AOO	2.5/1.1	5/1.2	10/2.84	NA	NA	-
	Gerberick et al. (2005)		2.5/1.3	5/1.1	10/2.5	25/10	50/17	+
Linalool alcohol	Gerberick et al. (2005)	AOO	25/2.5	50/4.8	100/8.3	NA	NA	+
	Data Submitted by D. Basketter, I. Kimber, and G. F. Gerberick		1/1.0	10/1.3	30/1.3	NA	NA	-
1-Chloro-2-dinitrobenzene	Gerberick et al. (2005)	AOO	0.01/1.5	0.025/1.8	0.05/2.4	0.1/8.9	0.25/38	+
	Data submitted by D. Germolec		0.01/1.17	0.03/1.12	0.05/1.93	0.1/1.95	0.25/7.10	+
Methyl salicylate	Gerberick et al. (2005)	AOO	1.0/1.0	2.5/1.1	5.0/1.6	10/1.4	20/0.9	-
	Data submitted by D. Germolec		1/0.86	2.5/1.19	5/1.16	10/1.41	20/1.72	-
Potassium dichromate	Gerberick et al. (2005)	DMSO	0.025/1.6	0.05/1.4	0.1/3.8	0.25/5.3	0.5/16.1	+
	Data submitted by D. Germolec		0.025/1.21	0.05/1.84	0.1/2.22	0.25/3.39	NA	+
	Ryan et al. (2002)		0.025/1.4	0.05/2.5	0.1/9.5	0.25/25.9	0.5/10.1	+

Abbreviations: AOO = Acetone Olive Oil; Conc. = Concentration tested; DMSO = Dimethylsulfoxide; NA = Not applicable since only three or four concentrations were tested; SI = Stimulation Index.

[1] - = non-sensitizer, + = sensitizer

- Based on available data (5 substances), 100% concordance in classification of substances as sensitizers or non-sensitizers was observed for 60% (3/5) of the substances.
- No additional studies were available to assess the reliability of the LLNA limit dose procedure.
- Since the LLNA limit dose procedure and traditional LLNA use identical protocols, and the datasets used to evaluate the accuracy of both procedures are similar, the intra- and inter-laboratory reliability of the LLNA limit dose procedure is expected to be the same as the traditional LLNA (see ICCVAM [1999a] for these statistics).

Independent Scientific Peer Review

A NICEATM-ICCVAM international independent scientific peer review panel met on March 4-6, 2008, to evaluate the validation status of the LLNA Limit Dose Procedure (announced in *Federal Register*, January 8, 2008; notice available at http://iccvam.niehs.nih.gov). A draft Background Review Document (ICCVAM, 2008a) and draft ICCVAM Recommendations (ICCVAM, 2008b) were reviewed by the Panel. The Panel's report is expected to be available by early May 2008, and will be available on the ICCVAM-NICEATM website, or can be obtained on request from NICEATM (niceatm@niehs.nih.gov).

Conclusions

Test Method Performance

- A retrospective analysis of data for 466 substances for the traditional LLNA was used to assess the performance of the limit dose procedure. Compared to the traditional LLNA, the LLNA limit dose procedure had an accuracy of 98.9% (461/466), a false positive rate of 0% (0/153), and a false negative rate of 1.6% (5/313).
- However, the LLNA Limit Dose Procedure does not provide dose response information, and an EC3 cannot be calculated. Therefore the Limit Dose Procedure should not be used for testing situations where dose response information is required.

Reduction in Animal Use

- Compared to the traditional LLNA, the LLNA limit dose procedure will reduce the number of animals used to assess skin sensitization.
- In the LLNA limit dose procedure, only the highest dose level of the test substance is evaluated in addition to the control groups, so the number of animals tested is decreased by at least 40%.

Cost Savings

- Since at least 40% fewer animals are tested in LLNA limit dose procedure, costs are expected to be proportionally lower than for the traditional LLNA.

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More information on ICCVAM and NICEATM can be accessed at: http://iccvam.niehs.nih.gov/.

Acknowledgements

ICCVAM and NICEATM gratefully acknowledge the following individuals and institutions who submitted data to NICEATM used for the evaluation of the LLNA limit dose procedure:

David Basketter, Ph.D., Unilever Safety and Environmental Assurance Centre, Sharnbrook, UK

Phil Botham, Ph.D., European Crop Protection Association, Brussels, Belgium

Eric Debruyne, Ph.D., Bayer CropScience SA, Sophia Antipolis, Cedex, France

G. Frank Gerberick, Ph.D., Procter and Gamble Company, Cincinnati, OH

Dori Germolec, Ph.D., National Toxicology Program, Research Triangle Park, NC, USA

Ian Kimber, Ph.D., University of Manchester, Manchester, UK

Michael J. Olson, Ph.D., GlaxoSmithKline, Research Triangle Park, NC, USA

Kirill Skirda, Ph.D., TNO Quality of Life, Delft, Netherlands

Peter Ungeheuer, Ph.D., European Federation for Cosmetic Ingredients, Frankfurt, Germany

Hans Werner Vohr, Ph.D., Bayer HealthCare, Wuppertal-Elberfeld, Germany

This poster was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences. ILS staff are supported by NIEHS contract N01-ES 35504.

The views expressed on this poster do not necessarily represent the official position of any federal agency.