Draft Updated Assessment of the Validity of the LLNA for Mixtures, Metals, and Aqueous Solutions

Addendum No. 1 to the ICCVAM Report: *The Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds (NIH Pub. No. 99-4494)*

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LIST OF ABBREVIATIONS AND ACRONYMS

ACD	Allergic contact dermatitis
AOO	Acetone: olive oil
BGIA	Berufsgenossenschaftliches Institut fur Arbeitsschutz (German Institute for Occupational Safety and Health)
BRD	Background Review Document
BT	Buehler Test
CASRN	Chemical Abstracts Service Registry Number
CESIO	Comite Europeen des Agents de Surface et de Leurs Intermediaires Organiques (European Committee of Surfactants and Their Organic Intermediates)
Conc.	Concentration tested
CPSC	U.S. Consumer Product Safety Commission
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EC3	Estimated concentration needed to produce a stimulation index of three
ECPA	European Crop Protection Association
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
EtOH	Ethanol
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
GCP	Good Clinical Practice
GHS	United Nations Globally Harmonized System for the Classification and Labelling of Chemicals
GLP	Good Laboratory Practice
g/mol	Grams per Mole
GP	Guinea pig
GPMT	Guinea Pig Maximization Test

GSK	GlaxoSmithKline
HCA	Hexyl cinnamic aldehyde
HMT	Human Maximization Test
HRIPT	Human Repeat Insult Patch Test
H_2O	Water
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IWG	Immunotoxicity Working Group
ISO	International Organization for Standardization
JaCVAM	Japanese Center for the Validation of Alternative Methods
K_{ow}	Octanol-water partition coefficient
LLNA	Local Lymph Node Assay
MeSH	Medical Subject Headings
n	Number
No.	Number
NA	Not available
NC	Not calculated
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIOSH	National Institute of Occupational Safety and Health
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
QRA	Quantitative Risk Assessment
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index
TG	Test Guideline
TNO	TNO Nutrition and Food Research (Dutch - No English translation)

102	U.K.	United Kingdom
103	U.S.	United States
104	vs.	Versus
105	w/v	Weight to volume ratio

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Preface

In 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in conjunction with the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) evaluated the validation status of the murine local lymph node assay (LLNA) as an alternative to guinea pig test methods for assessing the skin sensitization potential of substances. As described in the ICCVAM evaluation report[1], ICCVAM recommended that the LLNA could be used as a valid substitute for most testing situations. However, based on the lack of available data for aqueous solutions and mixtures and on discordant results for a limited number of studies with metals, ICCVAM recommended that these substances not be tested for skins sensitization using the LLNA.

Based on the ICCVAM recommendations, the ICCVAM member agencies that require the regulatory submission of skin sensitization data accepted the LLNA, with the identified limitations, as an alternative to the traditional guinea pig tests (Guinea Pig Maximization Test, Buehler Test). In 2002, the LLNA was adopted as Test Guideline 429 by the 30-member countries of the Organisation for Economic Co-operation and Development (OECD).

The information described in this addendum was compiled by ICCVAM in response to a nomination submitted in January 2007 by the U.S. Consumer Product Safety Commission for an assessment of the usefulness and limitations for the LLNA in testing mixtures, metals, and substances in aqueous solutions, among other activities related to the LLNA.

On May 17, 2007, NICEATM published a *Federal Register* (*FR*) notice (Vol. 72, No. 95, pp. 27815-27817[2]) requesting:

1. Public comments on the appropriateness and relative priority of the activities nominated by CPSC
2. Nominations of expert scientists to consider as members of a peer review panel

[1] ICCVAM (1999), available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel98.htm
[2] available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

3. Submission of data for the LLNA and/or any of the modified versions of the LLNA under consideration

After considering comments from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) on this nomination, ICCVAM assigned it a high priority, and directed NICEATM and the ICCVAM Immunotoxicity Working Group (IWG) to conduct a review of the current literature and an evaluation of the available data. ICCVAM and its IWG developed draft test recommendations based on this evaluation. An independent peer review panel (Panel) meeting will be convened to peer review the addendum and to evaluate the extent to which the information contained in the addendum support the draft recommendations. ICCVAM will consider the conclusions and recommendations of the Panel, along with comments received from the public and SACATM when developing a final addendum and final recommendations for each of the nominated activities.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We would also like to recognize the efforts of the individuals who contributed to the preparation of this addendum. These include David Allen, Ph.D., Thomas Burns, M.S., Neepa Choksi, Ph.D., Michael Paris, Eleni Salicru, Ph.D., Catherine Sprankle, Judy Strickland, Ph.D., and Doug Winters, M.S., of Integrated Laboratory Systems, Inc., the NICEATM Support Contractor, as well as the members of the ICCVAM IWG and the ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this final draft version. We also want to thank Raymond Tice, Ph.D., Deputy Director of NICEATM, for his contributions to this project. Finally, we want to recognize the excellent leadership of the IWG Co-chairs, Abigail Jacobs, Ph.D. (FDA) and Joanna Matheson, Ph.D. (CPSC).

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Executive Summary

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that the murine local lymph node assay (LLNA) could be used as a valid substitute for currently accepted guinea pig test methods to assess the skin sensitization potential of many, but not all types of substances. The recommendation was based on a comprehensive evaluation of 209 substances tested in the LLNA for which comparative guinea pig and/or human sensitization data were available (ICCVAM 1999; Sailstad et al. 2001). The evaluation included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA (ICCVAM 1999[1]).

ICCVAM forwarded recommendations to U.S. Federal agencies that the LLNA should be considered for regulatory acceptance or other non-regulatory applications for assessing the skin sensitization potential of substances, while recognizing that some testing situations would still require the use of traditional guinea pig test methods (ICCVAM 1999, Sailstad et al. 2001). The testing situations for which applicability of the LLNA had not been adequately demonstrated included the evaluation of metals or metal compounds. ICCVAM and the Panel also noted that there were insufficient data to support the testing of mixtures in the LLNA. Although not discussed in the original ICCVAM recommendations (ICCVAM 1999; Sailstad et al. 2001), the use of aqueous vehicles in the LLNA has also been cited as problematic, presumably due to the propensity of aqueous solutions to run off the ear during treatment.

The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline 429 [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and Sensitization [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effect Testing Guidelines on Skin Sensitization [EPA 2003]).

The information described in this addendum to the 1999 ICCVAM report was compiled by ICCVAM in response to a nomination[2] in January 2007 by the U.S. Consumer Product Safety Commission (CPSC) for a re-assessment of the applicability domain of the LLNA,

[1] available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel98.htm

among other activities related to the LLNA. This addendum provides an updated comprehensive review of available data and information regarding the current usefulness and limitations of the LLNA for assessing the skin sensitizing potential of mixtures, metals, and substances tested in aqueous solutions. The information is based on a retrospective review of traditional LLNA data that were either submitted as part of the original LLNA evaluation (ICCVAM 1999), extracted from peer-reviewed publications, or submitted to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in response to a *Federal Register* (*FR*) notice requesting available data and information (Vol. 72, No. 95, pages 27815-27817, May 17, 2007[3]).

The information contained in this addendum is based on a retrospective review of LLNA data derived from a current database of over 500 substances (including mixtures) tested in the LLNA. In the original ICCVAM evaluation of the LLNA (ICCVAM 1999), the performance of the LLNA was compared to 1) the results from guinea pig tests and 2) information about sensitizers in humans (e.g., human maximization test [HMT] results, substances used in human repeat insult patch test [HRIPT], clinical data), where available. This addendum updates the LLNA performance analyses for mixtures, metals, and substances tested in aqueous solutions when compared to human and guinea pig results.

Mixtures: The updated NICEATM LLNA database contains test results on 18 mixtures, 15 of which have comparative guinea pig data while none have comparative human data. In the guinea pig, six were classified as sensitizers and nine as non-sensitizers. Ten of the 15 mixtures are pesticides (i.e., herbicides, fungicides, insecticides) and four are dyes. Information on the product class for the remaining mixture was not identified. Information on the ingredients in the various mixtures is known for only one of the 15 mixtures. Information on physical form was available for five of the 15 mixtures; four are solids and one is a liquid. Among these 15 mixtures, in the LLNA, 11 were tested in an aqueous vehicle and four were tested in a non-aqueous vehicle. Compared to guinea pig, the LLNA has an accuracy of 53% (8/15), a sensitivity of 50% (3/6), a specificity of 56% (5/9), a false positive rate of 44%

[2] available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[3] available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

(4/9), and a false negative rate of 50% (3/6). When considering only aqueous mixtures with guinea pig data, six are sensitizers and five are non-sensitizers in the LLNA. For these mixtures, the LLNA has an accuracy of 64% (7/11), a sensitivity of 100% (2/2), a specificity of 56% (5/9), a false positive rate of 44% (4/9), and a false negative rate of 0% (0/2). When considering the four non-aqueous mixtures with comparative guinea pig data, the LLNA has an accuracy and a sensitivity of 25% (1/4) and a false negative rate of 75% (3/4)

Metals: A total of 17 metal compounds represented by 13 different metals are included in the updated NICEATM database. All 17 metal compounds had comparative human data and eight had comparative guinea pig data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, three times as nickel chloride, and once as a nickel (II) salt. Because nickel was classified as a sensitizer in four of these studies and as a non-sensitizer in the other four, a decision was made to exclude nickel compounds from the LLNA metals performance analysis.

For these remaining 14 metal compounds (13 metals), the LLNA had an accuracy of 86% (12/14), a sensitivity of 100% (9/9), a specificity of 60% (3/5), a false positive rate of 40% (2/5) and a false negative rate of 0% (0/9), when compared to human results. The two false positive compounds were copper chloride and zinc sulfate. All six of the metal compounds (six different metals with nickel compounds excluded) with comparative guinea pig test results were predicted as sensitizers by the LLNA. For these metal compounds, the LLNA had an accuracy of 83% (5/6), a sensitivity of 100% (5/5), a specificity of 0% (0/1), a false positive rate of 100% (1/1) and a false negative rate of 0% (0/5), when compared to guinea pig test results. When comparing the performance of the LLNA and the guinea pig tests, for the six metal compounds tested in all three species, to human results, the LLNA had an accuracy of 88% (7/8), a sensitivity of 100% (7/7), a specificity of 0% (0/1), a false positive rate of 100% (1/1) and a false negative rate of 0% (0/7); the accuracy of the guinea pig against the human remained the same as previously calculated.

Substances tested in aqueous solutions: A total of 21 substances tested in aqueous solutions are included in the updated NICEATM database. In the original ICCVAM evaluation of the validation status of the LLNA, substances tested in aqueous solutions were not analyzed separately (ICCVAM 1999). Among the 21 substances tested in aqueous solutions, 12 are

sensitizers and nine are non-sensitizers in the LLNA. The only product class represented by more than one aqueous solution (with six substances tested) was pesticides (i.e. herbicide, fungicides, insecticides).

Human data were available for four of the 21 substances tested in aqueous solutions with one being classified as a sensitizer and three as non-sensitizers by the LLNA. In comparison to the human data, the LLNA has an accuracy and sensitivity of 50% (1/2), and a false negative rate of 50% (2/4). Of these 21 substances tested in aqueous solutions, guinea pig data were available for six, which included two sensitizers and four non-sensitizers in the guinea pig. Based on the guinea pig test data, the LLNA has an accuracy of 50% (3/6), a sensitivity of 50% (1/2), a specificity of 50% (2/4), a false positive rate of 50% (2/4), and a false negative rate of 50% (1/2). Two substances tested in aqueous solutions (neomycin sulfate and propylene glycol) had data available from the LLNA, human tests, and guinea pig tests. One (neomycin sulfate) was false negative when LLNA data was compared to the guinea pig and human test results, while the other (propylene glycol) was false negative when the LLNA and guinea pig data were compared to human data.

1.0 Introduction

In February 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) received a submission from Drs. G. Frank Gerberick (Procter and Gamble, Cincinnati, United States [U.S.]), David Basketter (Unilever Safety and Environmental Assurance Centre, United Kingdom [U.K.]), and Ian Kimber (Syngenta Central Toxicology Laboratory, U.K.) requesting an evaluation of the validation status of the local lymph node assay (LLNA) as an alternative to the Guinea Pig Maximization Test (GPMT) and the Buehler Test (BT) for assessing skin sensitization potential. The submission summarized the performance (relevance and reliability) of the LLNA as compared to the GPMT and BT methods. An additional analysis was conducted by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to evaluate, where comparable data existed, the comparative performance of the LLNA and the guinea pig tests against sensitization results obtained in humans. An independent expert peer review panel (Panel) meeting was convened on September 17, 1998, to review the completeness of the submission, to determine whether the usefulness and limitations of the LLNA had been adequately described, and to decide whether its demonstrated performance supported recommending the LLNA as a stand-alone alternative to the GPMT and BT. The Panel also was asked to evaluate whether the LLNA offered advantages with regard to animal welfare considerations (i.e., refinement, reduction, or replacement[1]).

The Panel considered the performance of the LLNA to be similar to that of the GPMT and BT for identifying moderate to strong sensitizers. The Panel concluded that the LLNA did not accurately predict all weak sensitizers, nor did it adequately discriminate between strong skin irritants and skin sensitizers. The LLNA also produced false negative results with some metals. It was recommended that these issues be evaluated in future studies and workshops. Furthermore, data to support using the LLNA to test mixtures and substances tested in

[1] Refinement alternative is defined as a new or revised test method that refines procedures to lessen or eliminate pain or distress to animals, or enhances animal well-being; Reduction alternative is defined as a new or revised test method that reduces the number of animals required; Replacement alternative is defined as a new or revised test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate) (ICCVAM 1997).

aqueous solutions were not provided and the evaluation of pharmaceuticals was limited. Still, the Panel noted that when compared with the GPMT and BT methods, the LLNA appeared to provide equivalent prediction of risk for human allergic contact dermatitis (ACD), based on comparisons to available human data.

In addition, the Panel concluded that the LLNA could be considered a refinement alternative to the GPMT and BT, because the pain and distress due to sensitization associated with the guinea pig methods could be virtually eliminated by using the LLNA. ICCVAM agreed that the LLNA test method, when modified and used in accordance with the Panel report, can be used effectively for assessment of skin sensitization potential (ICCVAM 1999 [available in **Appendix A]**).

The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline 429 [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and Sensitization [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effect Testing Guidelines on Skin Sensitization [EPA 2003]).

NICEATM conducted this updated evaluation in response to a nomination[2] submitted to ICCVAM in January 2007 by the U.S. Consumer Product Safety Commission (CPSC). This addendum to the ICCVAM (1999) report contains an evaluation of the current database for the LLNA when used to test mixtures, metals, and substances in aqueous solutions in order to fill some of the data gaps identified in the original evaluation (see **Appendix A**).

The data summarized in this addendum are based on information obtained from the peer-reviewed scientific literature identified through online searches via PubMed and SCOPUS, through citations in publications, and in response to a *Federal Register* (*FR*) notice requesting LLNA, guinea pig, and/or human skin sensitization data and experience (Vol. 72, No. 95, pp. 27815-27817[3]). Key words used in the online searches for this evaluation were "LLNA" OR "Local Lymph Node" OR "Local lymph node" OR "local lymph node" AND

[2] available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[3] available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

345 (mixture* OR formula*)" OR ("metal* OR aqueous*)"; the last comprehensive search was
346 completed on January 15, 2008.

2.0 Substances Used for the Updated Evaluation of the Applicability Domain for the LLNA

The information summarized in this addendum is based on a retrospective review of LLNA data derived from a database of over 500 substances (including mixtures) tested in the LLNA and builds on the previous ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). For this evaluation, to minimize the complexity of the analysis, metal mixtures are not included in the analysis of mixtures and metal compounds, and aqueous solutions were restricted to those testing single substances. Mixtures were analyzed as one category and also separately as aqueous and non-aqueous mixtures. The reference database includes data for metal compounds from the original ICCVAM evaluation (**Appendix A**), data published since that evaluation, and data submitted in response to a request in the previously cited *FR* notice. Since an evaluation of the usefulness and limitations of mixtures and substances tested in aqueous solutions were not included in original ICCVAM validation (**Appendix A**), because no data on these substances were available, the reference database for these substances consists of data published since the original ICCVAM evaluation or submitted in response to the *FR* notice. **Table 2-1** provides information on the sources of the data and the rationale for the substances tested.

Two of the LLNA studies submitted in response to the *FR* notice (from Dr. Dori Germolec at NIEHS) used the Balb/C strain of mice rather than the CBA/J and CBA/Ca strains of mice recommended for the LLNA by ICCVAM (ICCVAM 1999, Dean et al. 2001, EPA 2003), and the OECD (2002). One of these, sodium metasilicate (an aqueous solution), did not have comparative GP or human data and thus was not included in the performance analysis. The other study was for potassium dichromate (a metal), which was positive in the LLNA, GP, and human. As there are 22 LLNA studies for potassium dichromate included in **Appendix C2**, all of which are positive, excluding this study would have no impact on the performance analysis for metals. Two other studies cited in Griem et al. (2003) used both male and female mice, but single experiments were limited to one sex. These data were included in the evaluation.

Table 2-1 Summary of Data Sources and Rationale for Substance Selection

Data Source	N	Substance Selection Rationale
ECPA	39	Plant protection products (i.e., pesticides) were evaluated in the LLNA with a novel vehicle to assess its usefulness
Basketter et al. (1994, 1996, 1999, 2005)	16	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Ryan et al. (2002)	11	Original research with known water soluble haptens and known skin sensitizers to assess the usefulness of a novel vehicle in the LLNA
E. Debruyne (Bayer Crop Science SA)	10	Original research on different pesticide types and formulations in the LLNA
Kimber et al. (1991, 1995, 2003)	9	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Gerberick et al. (2005)[1]	6	Compiled from previously conducted LLNA studies (from published literature and unpublished sources) on substances of varying skin sensitization potential
Bundesanstalt fur Arbeitsschutz und Arbeitsmedizin	4	Original LLNA research on different dye formulations
H.W. Vohr (BGIA)	4	Original LLNA research with epoxy resin components as part of a validation effort for non-radioactive versions of the LLNA
Basketter and Scholes (1992)[2]	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Gerberick et al. (1992)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
D. Germolec (NIEHS)	2	Substances were evaluated by NTP for skin sensitization potential in the LLNA
Lea et al. (1999)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
M.J. Olson (GlaxoSmithKline)	2	Pharmaceutical substances tested in the LLNA
Unilever (unpublished data)	2	Metal substances evaluated for skin sensitization potential in the LLNA
Basketter and Kimber (2006)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Goodwin et al. (1981)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Griem et al. (2003)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Kligman (1966)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
J. Matheson (CPSC)	1	Published LLNA data submitted electronically to NICEATM, as a reference
K. Skirda (CESIO - TNO Report V7217)	1	Data were provided by CESIO member companies for use in paper titled "Limitations of the LLNA as preferred test for skin sensitization: concerns about false positive and false negative test result"
Total	118	

Abbreviations: BGIA=Berufsgenossenschaftliches Institut fur Arbeitsschutz; CESIO=Comite Europeen des Agents de Surface et de Leurs Intermediaires Organiques; CPSC = Consumer Product Safety Commission; ECPA=European Crop Protection Association; LLNA=Local Lymph Node Assay; NICEATM=National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; NIEHS=National Institute of Environmental Health Sciences; TNO=TNO Nutrition and Food Research

[1]These data were evaluated by 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).

[2]These LLNA studies used both male and female mice, but single experiments were limited to one sex.

To the extent possible, **Appendices B1**, **C1**, and **D1** provide information on the physicochemical properties (e.g., physical form), Chemical Abstracts Service Registry Number (CASRN), and chemical class for each mixture, metal compound, and substance in an aqueous solution tested, respectively. This information was obtained from published reports, submitted data, or through literature searches.

When available, chemical classes for each substance were retrieved from the National Library of Medicine's ChemID Plus database. If chemical classes were not located, they were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system[1]. Some substances were assigned to more than one chemical class; however, no substance was assigned to more than three classes. One complex pharmaceutical intermediate was simply identified as a pharmaceutical substance.

The generic composition of one of the formulated products evaluated by the European Crop Protection Association (ECPA) using the LLNA is included in **Appendix E**. None of the active ingredients have been tested using the LLNA but the active ingredients are historic materials and have guinea pig data (personal communication by Dr Eric Debruyne, Bayer CropScience in France). Likewise, none of the inerts (e.g., surfactants, solvents, etc.) have been tested independently. The formulations for the remaining mixtures have been requested by NICEATM, but since some of the data is proprietary, it is not available at this time.

Of the 18 mixtures evaluated, 10 are pesticide formulations (i.e., herbicides, fungicides, insecticides) and four are dyes. Information on the product class for the remaining four mixtures has not been identified. Where information on physical form is available (10/18 mixtures), four are solids and six are liquids. Of the 13 metal compounds evaluated, one (potassium dichromate) is used in leather tanning and as an oxidizer in organic synthesis. Most of the remaining 12 metals in the analysis are used as catalysts, conductors of electricity, or for coating and plating. All of the metal compounds for which information on

[1] available at http://www.nlm.nih.gov/mesh/meshhome.html

411 physical form is identified are solids. Of the 21 substances tested in aqueous solutions
412 evaluated, six are pesticides (i.e., herbicide, fungicides, insecticides); this is the only product
413 class represented by more than one substance tested in an aqueous solution.

3.0 Comparative *In Vivo* Reference Data

The reference database for this evaluation includes results using currently accepted guinea pig test methods for skin sensitization (i.e., the GPMT and the BT) and human clinical studies and experience (e.g., human repeat insult patch test [HRIPT], human maximization test [HMT], case reports). In the absence of HRIPT or HMT data, the classification of a substance as a human sensitizer was based on the classification of the authors of the report. National and international test guidelines are available for each of these standardized tests and are thus described in detail elsewhere (OECD 1992, EPA 2003).

Ongoing efforts are being made by NICEATM to obtain the original records for all of the reference data used in this evaluation. Ideally, all data supporting the validity of a test method should be obtained and reported from animal studies conducted in accordance with Good Laboratory Practice (GLP) guidelines (OECD 1998; EPA 2006a, 2006b; FDA 2007a). Equally, data based on human studies should be conducted in compliance with Good Clinical Practices (GCP) guidelines (ICH 1996). Both sets of guidelines provide an internationally standardized procedure for the conduct of studies, reporting requirements, archival of study data and records, and information about the test protocol, in order to ensure the integrity, reliability, and accountability of a study.

The extent to which the human or guinea pig studies were compliant with GCP or GLP guidelines, respectively, is based on the information provided in published and submitted reports. The GP data obtained from E. Debruyne (Bayer CropScience SA) and P. Botham (ECPA) were reportedly conducted according to GLP guidelines. None of the published references from which GP or human data were obtained have GCP or GLP information specified.

4.0 LLNA Data and Results

The data used for this evaluation were obtained from 25 sources (**Table 2-1**). No new LLNA studies were conducted for this evaluation (see **Section 2.0**). Where available, specific information including name, CASRN, physicochemical properties (e.g., molecular weight, Log K_{ow}), chemical class[1] and data source are indicated for each mixture, metal compound, and substances tested in an aqueous solution (**Appendices B1, C1,** and **D1**, respectively). The concentration tested, along with calculated stimulation index (SI) and/or EC3 (the concentration that induces an SI of 3) values, are provided in **Appendices B2**, **C2,** and **D2** for mixtures, metal compounds, and substances tested in an aqueous solution, respectively. Individual components and concentrations of the mixtures and substances tested in an aqueous solution submitted by Bayer have been requested but due to confidential and proprietary issues they have only been able to provide the generic composition for four formulated products (one mixture, three substances tested in an aqueous solution) at this time (see **Section 2.0**). Furthermore, other than the information provided in the submitted data, no additional attempt was made to identify the source or purity of the test substance.

LLNA classification as to whether a substance was a sensitizer or a non-sensitizer was based on study data extracted from the sources listed in **Table 2-1** and **Appendices B1**, **C1**, and **D1,** with two exceptions. Classification of ammonium tetrachloroplatinate and gold (III) chloride (both of which are metal compounds) as sensitizers by the LLNA was based on published reference classifications (Basketter and Scholes 1992, Basketter et al. 1999) and not on actual LLNA data.

The LLNA data included in the ICCVAM (1999) database (**Appendix A**) were reviewed during the original evaluation. However, the availability of the original data for the other studies included in this evaluation has not yet been established for all data sources. Additionally, coding of substances to avoid potential scoring bias was not described in the previous evaluation of 209 substances (ICCVAM 1999; **Appendix A**) or for any of the newly obtained studies used in this evaluation.

[1] Chemical classes were assigned by NICEATM based on the classification of the National Library of Medicine's Medical Subject Heading (available at http://www.nlm.nih.gov/mesh/meshhome.html).

5.0 Accuracy of the LLNA: Updated Applicability Domain

The ability of the LLNA to correctly identify mixtures, metal compounds, and substances tested in aqueous solutions as potential skin sensitizers was evaluated when compared to human and guinea pig data. The classification of mixtures, metal compounds, and substances tested in aqueous solutions and the relevant data for each substance is located in **Appendices B2**, **C2**, and **D2**, respectively. For comparison purposes, the performance of the LLNA database reported in the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**), is included in **Tables 5-1** to **5-3**.

5.1 Testing of Mixtures

The original ICCVAM LLNA report (ICCVAM 1999) did not include an analysis on the ability of the LLNA to predict the skin sensitizing potential of mixtures, because data were not available for that evaluation (**Appendix A**). The current LLNA database contains data on 18 mixtures, 15 of which have corresponding guinea pig sensitization data while none had corresponding human sensitization data. Each mixture is tested in either an aqueous vehicle or a non-aqueous vehicle, and no data is available for mixtures that were tested in both. Thus, of the 15 mixtures with corresponding guinea pig data, 11 are aqueous mixtures and four are non-aqueous mixtures (**Appendix B2**). In this analysis, all aqueous mixtures contained at least 20% water, while non-aqueous mixtures contained no water. The qualitative formulation for one of the mixtures included in this analysis are known and provided in **Appendix E**.

Among the 15 mixtures with comparative guinea pig data, six are classified as sensitizers and nine as non-sensitizers in the guinea pig. Compared to these guinea pig data, the LLNA has an accuracy of 53% (8/15), a sensitivity of 50% (3/6), a specificity of 56% (5/9), a false positive rate of 44% (4/9), and a false negative rate of 50% (3/6) (**Table 5-1**). When considering only aqueous mixtures with guinea pig data, the LLNA has an accuracy of 64% (7/11), a sensitivity of 100% (2/2), a specificity of 56% (5/9), a false positive rate of 44% (4/9), and a false negative rate of 0% (0/2) (**Table 5-1**). When considering the four non-aqueous mixtures with comparative guinea pig data, the LLNA has an accuracy and a sensitivity of 25% (1/4) and a false negative rate of 75% (3/4) (**Table 5-1**).

Table 5-1 Evaluation of the Performance of the LLNA in Testing Mixtures

Comparison[1]	n[2]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]
LLNA vs. GP[4] (All Mixtures)	15	53	8/15	50	3/6	56	5/9	44	4/9	50	3/6
LLNA vs. GP[4] (Aqueous Mixtures)	11	64	7/11	100	2/2	56	5/9	44	4/9	0	0/2
LLNA vs. GP[4] (Non-Aqueous Mixtures)	4	25	1/4	25	1/4	-	0/0	-	0/0	75	3/4
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[5]											
LLNA vs. GP[4]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[6]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[4] vs. Human[6]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations: GP = Guinea pig skin sensitization outcomes; LLNA = Local Lymph Node Assay; No. = Number.
Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method; Sensitivity = the proportion of all positive substances that are classified as positive; Specificity = the proportion of all negative substances that are classified as negative; False negative rate: the proportion of all positive substances that are falsely identified as negative; False positive rate = the proportion of all negative substances that are falsely identified as positive.
[1]This accuracy analysis is only for mixtures that have LLNA data and either corresponding guinea pig or human data; none of the mixtures analyzed had human data, so a comparison between LLNA vs. human and LLNA vs. GP is not included.
[2]n = Number of substances included in this analysis.
[3]The data on which the percentage calculation is based.
[4]GP refers to outcomes obtained by studies conducted using either the Guinea Pig Maximization Test or the Buehler Test.
[5]For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**) showing the overall performance of the LLNA vs. guinea pig and human, and guinea pig versus human is included here.
[6]Human refers to outcomes obtained by studies conducted using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

As mentioned previously, since comparative human data are not available for any of the mixtures analyzed, an evaluation of mixtures in the LLNA compared to human performance could not be assessed. For the same reason, an evaluation of guinea pig versus human outcomes is also not possible. Also, no mixtures were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**), so these data and analyses cannot be compared to previously considered data.

5.2 Testing of Metal Compounds

The ICCVAM LLNA report (ICCVAM 1999) includes a summary on the ability of the LLNA to predict the skin sensitizing potential of 11 metal compounds, representing 10 different metals (**Appendix A**). In this addendum, the original ICCVAM analysis has been updated to include a total number of 17 metal compounds, representing 13 different metals, with corresponding human and/or guinea pig data (**Appendix C**). To reduce the complexity of the analysis, mixtures containing metals were not classified as metal compounds in this evaluation. Among these 17 metal compounds, 14 were tested in an aqueous vehicle, a non-

aqueous vehicle, or both. The vehicle in which the three remaining metal compounds (i.e. cobalt chloride, cobalt sulfate, and nickel (II) salts) were tested in was not specified (**Appendix C2**). Similar to mixtures (**Section 5.1**), aqueous vehicles contained at least 20% water, while a non-aqueous vehicle contains no water.

All 17 metal compounds had comparative human data and eight had comparative guinea pig data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, three times as nickel chloride, and once as a nickel (II) salt. Because nickel was classified as a sensitizer in four of these studies and as a non-sensitizer in the other four, a decision was made to exclude nickel compounds from the LLNA metals performance analysis.

Of the 14 remaining metal compounds (13 metals) tested in the LLNA and with human data, nine are sensitizers and five are non-sensitizers in humans. For these 14 metal compounds, the LLNA has an accuracy of 86% (12/14), a sensitivity of 100% (9/9), a specificity of 60% (3/5), a false positive rate of 40% (2/5) and a false negative rate of 0% (0/9), when compared to human results (**Table 5-2**). For the six metal compounds (after excluding nickel compounds) with guinea pig data (five sensitizers and one non-sensitizer in the guinea pig), the LLNA has an accuracy of 83% (5/6), a sensitivity of 100% (5/5), a specificity of 0% (0/1), a false positive rate of 100% (1/1) and a false negative rate of 0% (0/5), when compared to guinea pig test results (**Table 5-2**) (**Appendix C2**).

Furthermore, all six of the 14 metal compounds with guinea pig data have human data for comparison and there is a chemical-by-chemical match in classification between the guinea pig and human outcomes (**Table 5-2**). In contrast, the LLNA incorrectly identified one the human non-sensitizing metal compounds as a sensitizer. For comparative purposes, the corresponding performance of the LLNA in predicting the human response for these same six metal compounds is also provided in **Table 5-2**.

Table 5-2 Evaluation of the Performance of the LLNA in Testing Metal Compounds

Comparison	n[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
All Metal Compounds (Aqueous and Non-Aqueous Vehicles)											
LLNA vs. GP[3]	6	83	5/6	100	5/5	0	0/1	100	1/1	0	0/5
LLNA vs. Human[4]	14	86	12/14	100	9/9	60	3/5	40	2/5	0	0/9
GP[3] vs. Human[4]	6	100	6/6	100	5/5	100	1/1	0	0/1	0	0/5
LLNA vs. Human[4] for the same GP metal compounds	6	83	5/6	100	5/5	0	0/1	100	1/1	0	0/5
Metal Compounds Tested in Aqueous Vehicles[5]											
LLNA vs. GP[3]	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
LLNA vs. Human[4]	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
GP[3] vs. Human[4]	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
Metal Compounds Tested in Non-Aqueous Vehicles											
LLNA vs. GP[3]	5	80	4/5	100	4/4	0	0/1	100	1/1	0	0/4
LLNA vs. Human[4]	12	92	11/12	100	7/7	80	4/5	20	1/5	0	0/7
GP[3] vs. Human[4]	5	100	5/5	100	4/4	100	1/1	0	0/1	0	0/4
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[6]											
LLNA vs. GP[3]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[4]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[3] vs. Human[4]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations: GP = Guinea pig skin sensitization outcomes; LLNA = Local Lymph Node Assay; No. = Number.
Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method; Sensitivity = the proportion of all positive substances that are classified as positive; Specificity = the proportion of all negative substances that are classified as negative; False negative rate: the proportion of all positive substances that are falsely identified as negative; False positive rate = the proportion of all negative substances that are falsely identified as positive.
[1] n = Number of substances included in this analysis.
[2] The data on which the percentage calculation is based.
[3] GP refers to outcomes obtained by studies conducted using either the Guinea Pig Maximization Test or the Buehler Test.
[4] Human refers to outcomes obtained by studies conducted using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.
[5] All the metal compounds tested in an aqueous vehicle were also tested in a non-aqueous vehicle.
[6] For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**) showing the overall performance of the LLNA vs. guinea pig and human, and guinea pig versus human is included here.

Of the six metal compounds with guinea pig data, the vehicle is known for five of the six compounds. Four of these metal compounds were tested only in a non-aqueous vehicle, while one was tested in both an aqueous and non-aqueous vehicle. Thus, when considering only the metal compound with guinea pig data that was tested in an aqueous vehicle, it was a sensitizer in the LLNA and the LLNA correctly classified it compared to the guinea pig data.

566 This resulted in an accuracy and sensitivity of 100% (1/1) and a false negative rate of 0%
567 (0/1) (**Table 5-2**). All of the five metal compounds with comparative guinea pig data tested
568 in a non-aqueous vehicle are also classified as sensitizing in the LLNA. Compared to guinea
569 pig data, the LLNA correctly classifies four of the five non-aqueous metal compounds. This
570 results in an accuracy of 80% (4/5), a sensitivity of 100% (4/4), a specificity of 0% (0/1), a
571 false positive rate of 100% (1/1) and a false negative rate of 0% (0/4) (**Table 5-2**).

572 Of the 14 metal compounds with human data, the vehicle is known for 12 of the 14
573 compounds. Eleven of these metal compounds were tested only in a non-aqueous vehicle,
574 while one was tested in both an aqueous and non-aqueous vehicle. Thus, when considering
575 only the metal compound with human data that was tested in an aqueous vehicle, the LLNA
576 correctly classified it as a sensitizer compared to the human data (**Table 5-2**). In contrast, of
577 the 12 metal compounds with comparative human data tested in a non-aqueous vehicle, eight
578 are classified as sensitizers and the remaining four are non-sensitizers in the LLNA.
579 Compared to human data, the LLNA correctly classifies 11 of the 12 non-aqueous metal
580 compounds. This results in an accuracy of 92% (11/12), a sensitivity of 100% (7/7), a
581 specificity of 80% (4/5), a false positive rate of 20% (1/5) and a false negative rate of 0%
582 (0/7) (**Table 5-2**).

583 Potassium dichromate was the one metal compound with comparative guinea pig and human
584 data that was tested in both an aqueous and non-aqueous vehicle. Vehicle information was
585 available for 20 of the 22 LLNA studies included in this analysis on potassium dichromate,
586 indicating that it was tested six times in an aqueous vehicle (i.e., 1% Pluronic L92) and 14
587 times in a non-aqueous vehicle (dimethylformamide [DMF] or DMSO). In all cases, it was
588 found to be sensitizing by the LLNA regardless of the vehicle used.

589 For the purpose of this addendum, a case-by-case analysis was carried out to determine
590 whether the overall LLNA classification for each metal compound is as a sensitizer or a non-
591 sensitizer. In most cases, the majority result determined the overall LLNA skin sensitizing
592 classification for each metal compound. In instances where there were an equal number of
593 reports classifying the metal compound as sensitizing or non-sensitizing, the most severe
594 classification was used. For instance, for zinc sulfate, LLNA data from two studies are
595 considered in this evaluation report (ICCVAM 1999 [**Appendix A**] and Basketter et al.

1999). Zinc sulfate is classified as a sensitizer in ICCVAM 1999 (neither the vehicle nor the raw data were included) whereas Basketter et al. (1999) classified zinc sulfate as a non-sensitizer when using DMSO as the vehicle (SI = 2.3 at 25%). For the purposes of this evaluation, to be conservative, zinc sulfate is classified as a sensitizer (**Appendix C2**).

Based on the data compiled for this evaluation, the LLNA classification for nine of the 11 metal compounds evaluated in the 1999 ICCVAM report remained the same in this evaluation because either no new data were available or classifications based on new data were consistent with the original classification (**Appendix A**). For the remaining two metal compounds (nickel chloride and nickel sulfate), additional LLNA data were available, but as described above, discordant results with nickel compounds in eight different LLNA studies precluded a definitive classification and it was therefore excluded from this analysis.

5.3 Testing of Substances in Aqueous Solutions

The ICCVAM report (ICCVAM 1999) did not include an analysis of the ability of the LLNA to predict the skin sensitizing potential of substances tested in aqueous solutions, because data were not available for that evaluation (**Appendix A**). In this addendum, the ICCVAM 1999 report has been updated to include a total of 21 unique substances tested in aqueous solutions from 47 LLNA studies with corresponding human and/or guinea pig data (**Appendix D**). In this analysis, an aqueous solution is defined as a substance tested in a vehicle containing at least 20% water. The group of substances analyzed for this section of the addendum does not include any known mixtures or metal compounds tested in aqueous vehicles, as they have instead been included in the analyses discussed in **Sections 5.1** and **5.2** (mixtures and metal compounds, respectively).

Among the 21 substances tested in aqueous solutions, human data were available for only four (3 sensitizers/1 non-sensitizer in humans). Of these four, two were correctly identified by the LLNA when compared to human data. Consequently, for these four substances tested in aqueous solutions, the LLNA has an accuracy of 50% (2/4), a sensitivity of 33% (1/3), a specificity of 100% (1/1), a false positive rate of 0% (0/1), and a false negative rate of 67% (2/3) (**Table 5-3**).

Table 5-3 Evaluation of the Performance of the LLNA in Testing Aqueous Solutions

Comparison	n[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
Aqueous Solutions											
LLNA vs. GP[3]	6	50	3/6	50	1/2	50	2/4	50	2/4	50	1/2
LLNA vs. Human[4]	4	50	2/4	33	1/3	100	1/1	0	0/1	67	2/3
GP[3] vs. Human[4]	2	50	1/2	50	1/2	-	0/0	-	0/0	50	1/2
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[5]											
LLNA vs. GP[3]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[4]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[3] vs. Human[4]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations: GP = Guinea pig skin sensitization outcomes; LLNA = Local Lymph Node Assay; No. = Number.
Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method; Sensitivity = the proportion of all positive substances that are classified as positive; Specificity = the proportion of all negative substances that are classified as negative; False negative rate: the proportion of all positive substances that are falsely identified as negative; False positive rate = the proportion of all negative substances that are falsely identified as positive

[1] n = Number of substances included in this analysis.

[2] The data on which the percentage calculation is based.

[3] GP refers to outcomes obtained by studies conducted using either the Guinea Pig Maximization Test or the Buehler Test.

[4] Human refers to outcomes obtained by studies conducted using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

[5]For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**) showing the overall performance of the LLNA vs. guinea pig and human, and guinea pig versus human is included here.

Guinea pig data were available for six (2 sensitizers/4 non-sensitizers in the guinea pig) of the 21 substances tested in aqueous solutions. Thus, the LLNA has an accuracy of 50% (3/6), a sensitivity of 50% (1/2), a specificity of 50% (2/4), a false positive rate of 50% (2/4), and a false negative rate of 50% (1/2) (**Table 5-3**). There were two substances tested in aqueous solutions with comparative human and guinea pig data. Of these, one (propylene glycol) was false negative in guinea pig and one (neomycin sulfate) was correctly identified as positive compared to human results (**Table 5-3**). These two substances tested in aqueous solutions LLNA are false negative compared to human results.

For the purpose of this addendum, a case-by-case analysis was carried out to determine whether the overall LLNA classification for each substance tested in aqueous solutions is as a sensitizer or a non-sensitizer. In most cases, the majority result determined the overall LLNA skin sensitizing classification for each substance (i.e., oxyfluorfen EC). In instances where there were an equal number of reports classifying the aqueous solution as sensitizing or non-sensitizing, the overall LLNA classification took into account the concentrations tested or, if the studies appeared to be equal, the most severe classification was used. For instance, in one

of two LLNA studies, 1.4-dihydroquinone (in ACE/saline [1:1]) is classified as a skin sensitizer resulting in an EC3 value of 1.3%. In the other, which also used ACE/saline (1:1) as the vehicle, 1.4-dihydroquinone is classified as a non-sensitizer (SI = 1.9 at 1%). However, because the highest concentration tested in the negative study (1%) was below the EC3 concentration in the positive study (10%), 1.4-dihydroquinone is classified as a sensitizer in this evaluation (**Appendix D2**).

Because no substances tested in aqueous solutions were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**), these data and analyses cannot be compared to previously considered data.

6.0 LLNA Data Quality

Based on the available information, the published papers, and data submissions, information on compliance with GLP guidelines was available for data obtained from Gerberick et al. (2005), H.W. Vohr (BGIA), E. Debruyne (Bayer CropScience SA), P. Botham (ECPA), Bundesanstalt fur Arbeitsschutz und Arbeitsmedizin, and D. Germolec (NIEHS).

A formal assessment of the quality of the remainder of the LLNA data considered here was not feasible. The published data on the LLNA were limited to tested concentrations and calculated SI and EC3 values. Auditing the reported values would require obtaining the original individual animal data for each LLNA experiment, which have been requested, but not yet obtained. However, many of the studies were conducted according to GLP guidelines, which implies that an independent quality assurance audit was conducted. The impact of any deviations from GLP guidelines cannot be evaluated for the data reviewed here, since no data quality audits was obtained.

As noted in **Section 5.0,** the original records were not obtained for the studies included in this evaluation. Data were available for several of the substances included in the ICCVAM (1999) evaluation and thus some of the raw data for these substances were available for review.

7.0 Other Scientific Reports and Reviews

A search of Medline, PubMed, and Toxline resulted in 40 published reports relevant to the applicability domain of the LLNA and the use of the LLNA for testing mixtures, metals and aqueous solutions for skin sensitizing potential. Of these reports, 23 have been published since the 1999 ICCVAM report on the LLNA. Included below are the reports most relevant to the evaluation included in this addendum, with the most salient points summarized for each.

7.1 Basketter et al. (1999)

Basketter et al. (1999) used the LLNA to evaluate the skin sensitization potential of 13 metal salts. For the purposes of their evaluation, eight of the 13 metals were considered to be human sensitizers. Their results show that the LLNA had an accuracy of 85% (11/13), sensitivity 88% (7/8), specificity of 80% (4/5), false negative rate of 12% (1/8), and false positive rate of 20% (1/5). Nickel chloride (tested up to 5% in DMSO) was false negative in the LLNA based on an SI $\leq$ 2.4. Copper chloride (tested up to 5% in DMSO) was false positive in the LLNA based on an SI $\geq$ 8.1. The authors concluded that these data support the potential utility of the LLNA for testing metal contact allergens.

7.2 Wright et al. (2001)

The authors investigate the influence of application vehicle on sensitizing potency, using the LLNA to examine the activity of four recognized human contact allergens: isoeugenol and cinnamic aldehyde and two fragrance chemicals; 3-dimethylaminopropylamine (a sensitizing impurity of cocamidopropyl betaine, a surfactant used in shower gel) and dibromodicyanobutane (the sensitizing component of Euxyl K 400, a preservative used in cosmetics). The four chemicals were applied in each of seven different vehicles (acetone: olive oil [4 : 1]; dimethyl sulfoxide: methyl ethyl ketone; dimethylformamide; propylene glycol; and both 50:50 and 90:10 mixtures of ethanol and water). It was found that the vehicle in which a chemical is presented to the epidermis can have a marked effect on sensitizing activity. EC3 values ranged from 0.9 to 4.9% for isoeugenol, from 0.5 to 1.7% for cinnamic aldehyde, from 1.7 to > 10% for dimethylaminopropylamine and from 0.4 to 6.4% for dibromodicyanobutane. These authors confirm that the vehicle in which a chemical is

encountered on the skin has an important influence on the relative skin sensitizing potency of chemicals and may have a significant impact on the acquisition of allergic contact dermatitis. The data also demonstrate the utility of the LLNA as a method for the prediction of these effects and thus for the development of more accurate risk assessments.

7.3 Ryan et al. (2002)

Ryan et al. (2002) describe data on Pluronic® L92 (L92), a water-based vehicle, that possessed better skin wetting properties than water alone and assessed its performance relative to other solvents in the LLNA using aqueous soluble haptens. Based on their results, the authors determined that identification of sensitization hazard of aqueous soluble materials using the LLNA, DMF and DMSO were the preferred vehicles. However, if a test material is not soluble in DMF or DMSO, or if higher test concentrations can be achieved in an aqueous vehicle, then 1% L92 may provide a better alternative to water alone in terms of improved assay performance.

7.4 Griem et al. (2003)

The authors propose a quantitative risk assessment methodology for skin sensitization aimed at deriving 'safe' exposure levels for sensitizing substances. In their analysis they used cinnamic aldehyde and nickel as examples of how they apply their risk assessment proposal to sensitizing substances. In their discussion of nickel, they reference data supporting that nickel is an allergen with a relatively low sensitizing potency, but a high prevalence in the general population (Kligman 1966, Vandenberg and Epstein 1963). Consequently, as in humans, nickel salts (i.e. nickel chloride and nickel sulfate) are weak sensitizers in animals and often give negative results in standardized tests (e.g., LLNA). Clinical experience in humans indicates that nickel allergy preferentially develops after nickel exposure on irritated or inflamed, but not on healthy skin (Kligman 1966, Vandenberg and Epstein 1963). Similarly, previously false negative results with nickel salts in the mouse LLNA could recently be overcome by the addition of a detergent (1% surfactant in water) to the nickel test solution (Ryan et al. 2002).

7.5 Hostynek and Maibach (2003 and 2004)

In these two review papers, the authors consider reports of immediate and delayed type immune reactions to cutaneous or systemic exposure to copper in humans. They mention that the electropositive copper ion is potentially immunogenic due to its ability to diffuse through biological membranes to form complexes in contact with tissue protein. Reports of immune reactions to copper include ACD, immunologic contact urticaria (ICU), systemic allergic reactions (SAR) and contact stomatitis (STO). They state that considering the widespread use of copper intrauterine devices (IUDs) and the importance of copper in coinage, items of personal adornment and industry, unambiguous reports of sensitization to the metal are extremely rare, and even fewer are the cases, which appear clinically relevant. Reports of immune reactions to copper mainly describe systemic exposure from IUDs and prosthetic materials in dentistry, implicitly excluding induction of the hypersensitivity from contact with the skin as a risk factor. Based on predictive guinea pig test and the LLNA, copper has a low sensitization potential. The authors then provide a diagnostic algorithm that might clarify the frequency of copper hypersensitivity.

7.6 Lalko et al. (2006)

In the fragrance industry, mixtures are commonly found and include oils, which may contain naturally occurring contact sensitizers. Lalko et al. (2006) describe their studies where they used the LLNA to evaluate the dermal sensitization potential of basil, citronella, clove leaf, geranium, litsea cubeba, lemongrass, and palmarosa oils. Three of the major components--citral, eugenol, and geraniol--were included to investigate any difference in sensitization potential arising from their exposure in a mixture. Citronella and geranium oils were negative. The individual components citral, eugenol and geraniol resulted in EC3 values of 6.3%, 5.4% and 11.4%, respectively. In general, the potency of each essential oil did not differ significantly from that observed for its main individual component.

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