Revised Draft 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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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
CCA	Chromated copper arsenate
CESIO	Comite Europeen des Agents de Surface et de Leurs Intermediaires Organiques (European Committee of Surfactants and Their Organic Intermediates)
CoDEC	Cobalt diethyldithiocarbamate
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
EtOH	Ethanol
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
g/L	Grams per liter
GP	Guinea pig
GPMT	Guinea pig maximization test
GSK	GlaxoSmithKline
GST	Gold sodium thiosulfate
HMT	Human Maximization Test
HRIPT	Human Repeat Insult Patch Test
H_2O	Water
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
IUD	Intrauterine device
IWG	Immunotoxicity Working Group
K_{ow}	Octanol-water partition coefficient
LLNA	Local lymph node assay
MeSH	Medical subject headings
MEST	Mouse ear swelling test
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
TEDCD	Tetraethyldicarbamoyl disulfide
TETD	Tetraethylthiuram disulfide
TG	Test Guideline
TNO	TNO Nutrition and Food Research (Dutch - No English translation)
U.K.	United Kingdom
U.S.	United States
vs.	Versus
w/v	Weight to volume ratio
Veh.	Vehicle
ZDEC	Zinc diethyldithiocarbamate

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Preface

In 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999). ICCVAM concluded that the LLNA (referred to herein as the "traditional LLNA") provided several advantages compared to the guinea pig method, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for ACD testing. It is now commonly used around the world.

However, as described in the ICCVAM evaluation report[1], 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 skin sensitization potential 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 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) to reevaluate the usefulness and limitations of the LLNA for testing mixtures, metals, and substances in aqueous solutions, among other activities related to the LLNA. ICCVAM assigned the activity a high priority, and established the ICCVAM Immunotoxicity Working Group (IWG) to work with NICEATM to review the current literature and evaluate available data to assess the status of the LLNA applicability domain. A comprehensive draft addendum provided the information, data and analyses supporting the validation status of the LLNA applicability domain. IICVAM also developed draft test method recommendations for

[1] ICCVAM (1999), available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel98.htm

the LLNA applicability domain regarding usefulness and limitations, test method protocol, performance standards and future studies.

NICEATM and ICCVAM provided the draft addendum and draft recommendations to an international independent scientific peer review panel for their consideration at a public meeting on March 4-6, 2008. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website[2]. Both ICCVAM and the Panel concluded that, due to the limitations associated with the available database for mixtures (i.e., unknown formulae, lack of human data), more data were needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures could be made. The Panel also stated that the term "mixtures" was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations that were being examined. Public comments at the meeting revealed that additional relevant data from LLNA studies with pesticide formulations and other products were available, which had not previously been provided in response to earlier requests for data. The Panel recommended that NICEATM obtain additional existing data that was not available to the Panel, and reanalyze the performance of the LLNA for testing pesticide formulations and other products. NICEATM subsequently obtained additional data and prepared this revised addendum. ICCVAM also prepared revised draft test method recommendations based on the revised addendum. This revised draft addendum addresses the validation database for the LLNA applicability domain.

The Panel will meet to consider the revised addendum and to evaluate the extent to which the available information supports the revised ICCVAM draft test method recommendations. ICCVAM will consider the conclusions and recommendations of the Panel, along with comments received from the public and SACATM, and finalize the addendum and test method recommendations. These will then be forwarded to Federal agencies for acceptance decisions where appropriate.

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, including the following staff from the NICEATM Support Contractor, Integrated Laboratory Systems, Inc.: David Allen, Ph.D.,

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[2] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm

Executive Summary

Background

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the murine local lymph node assay (LLNA) is 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. ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf). 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 Organization for Standardization [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]).

In 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM (Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the nominated activities was an assessment of the validation status of the LLNA applicability domain. The information described in the original and this revised addendum was compiled by ICCVAM and NICEATM in response to this nomination.

This addendum provides a revised 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 Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in response to a *Federal Register* notice requesting available data and information (Vol. 72, No. 95, pages 27815-27817, May 17, 2007[3]).

Revisions to the NICEATM-ICCVAM Evaluation of the LLNA Applicability Domain

NICEATM and ICCVAM convened an independent scientific peer review panel meeting on March 4-6, 2008. The Panel peer reviewed the draft addendum and commented on the extent that it supports the draft ICCVAM test method recommendations on the usefulness and limitations of the LLNA regarding the applicability domain. Both ICCVAM and the Panel concluded that, due to the limitations associated with the available database for mixtures (i.e., unknown formulae, lack of human data), more data were needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures could be made[4]. The Panel also stated that the term "mixtures" was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations that are being examined (ICCVAM 2008).

Public comments at the meeting revealed that additional relevant data from LLNA studies with pesticide formulations and other products were available that had not previously been provided in response to earlier requests for data. The Panel recommended that the additional data be obtained by NICEATM and that a reanalysis of the performance of the LLNA for testing pesticide formulations and other products be conducted. In response to this recommendation, NICEATM obtained additional LLNA data and, in some cases, corresponding reference test method data (i.e., guinea pig test and/or human data) (ICCVAM 2008). These additional data were used to revise the evaluation of the LLNA for testing pesticide formulations and other products[5](**Section 5.1**) and for testing substances in aqueous solutions (**Section 5.3**). No new LLNA data were received for LLNA tests with metals, therefore this evaluation remains unchanged (**Section 5.2**).

[3] available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf
[4] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel08.htm
[5] Based on the Panel recommendation, this revised addendum does not refer to "mixtures" as a type of substance tested, but rather specifies the types of products that were tested, where possible.

The changes to the existing database that resulted from any new data received subsequent to the release of the January 2008 draft addendum are summarized as follows:

- LLNA data and corresponding *in vivo* guinea pig test method data for 52 pesticide formulations were submitted by Dow AgroSciences.
- LLNA data for 28 pesticide formulations were submitted by Dupont Chemical Company.
- Detailed LLNA study results and corresponding human data for 12 fragrance ingredients were submitted by the Research Institute for Fragrance Materials. The summary results were originally published in Lalko and Api (2006).
- LLNA data for 48 medical device eluates were submitted by AppTec Laboratory Services.

These new data sources have been added to **Table 2.1**.

Validation Database

This revised draft addendum considers data for 140 additional substances compared with the January 2008 draft. The information contained in this addendum is now based on a retrospective review of LLNA data derived from a current database of over 600 substances (including pesticide formulations and other products) 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 results, substances used in human repeat insult patch test, clinical data), where available. This addendum updates the LLNA performance analyses for pesticide formulations and other products, metals, and substances tested in aqueous solutions when compared to human and guinea pig results.

Use of the LLNA for Testing Formulations and Other Products

In contrast with the January 2008 draft, which used the term “mixtures” to refer to multiple component substances, this revised draft addendum categorizes substances with multiple components according to product category.

Pesticide Formulations: The revised LLNA database contains data for 104 pesticide formulations. Among these formulations, 54% (56/104) were LLNA positive and 46% (48/104) were LLNA negative.

Seventy of the 104 pesticide formulations have LLNA and some type of associated guinea pig reference data. A total of 89 LLNA studies were performed using these 70 formulations. LLNA studies were conducted with either CBA/Ca or CBA/J (61/89) and/or BALBc (28/89) mouse strains. Six pesticide formulations were tested in multiple LLNA studies (25 studies total); 5/6 multiply-tested pesticide formulations had LLNA results in agreement, and 1/6 pesticide formulations produced discordant results (3 positive, 2 negative).

All of these 70 pesticide formulations (89/89 studies) were tested in the LLNA in aqueous 1% Pluronic L92, a surfactant and wetting agent that has been evaluated as an alternative aqueous-based vehicle for use in the LLNA (Boverhof et al. 2008, Ryan et al. 2002).

Twenty-two pesticide formulations had associated guinea pig data for the complete formulation, 46 pesticide formulations had guinea pig data for one or more of the active ingredients included in the complete formulation, and 14 pesticide formulations had guinea pig data for a substance related to an active ingredient or for a related formulation.

For 22 formulations for which there were guinea pig data, the LLNA classified 54% (12/22) of the formulations as sensitizers while the guinea pig tests classified only 14% (3/22) formulations as sensitizers. All three of the pesticide formulations identified as sensitizers in the guinea pig test were also identified as sensitizers in the LLNA. Overall, the LLNA and the guinea pig results were in agreement 54% of the time. The LLNA also identified an additional seven substances as sensitizers that were classified as nonsensitizers in the guinea pig test, an overprediction of 53% (10/19). Three of the LLNA studies for the 22 pesticide formulations were done in BALB/c mice. If these three studies are removed from the analysis, the LLNA and the guinea pig results were in agreement 58% (11/19) of the time, and the overprediction was 50% (8/16). There were no instances of underprediction for these 22 pesticide formulations. Human data are not available for these pesticide formulations to confirm their actual sensitization potential in humans.

Dyes: The current LLNA database contains data for six dyes for which there is LLNA and guinea pig data. Based on LLNA results for these six dyes, 50% (3/6) were sensitizers and 50% (3/6) were nonsensitizers. By comparison, based on guinea pig maximization test (GPMT) results, 83% (5/6) were sensitizers (when there were multiple calls in the GPMT, a most conservative call was used) and 17% (1/6) were nonsensitizers. The LLNA and the guinea pig results were in agreement 33% of the time. The overprediction for the LLNA was 100% (1/1) and the underprediction was 60% (3/5).

Fragrance Ingredients: The current LLNA database also contains data for 12 fragrance ingredients (essential oils and absolutes) for which there are comparative LLNA and human data. Essential oils are oils derived from a natural source using steam or pressure. Absolutes are purified extracts from natural products. Both essential oils and absolutes are substances comprised of more than one component. Based on LLNA results for these fragrance ingredients, 75% (9/12) were sensitizers and 25% (3/12) nonsensitizers. However, based on human clinical studies, only 33% (4/12) of these substances tested as sensitizers. Therefore, compared to human outcomes for these 12 substances, the LLNA was able to identify three out of four of the substances that were positive in human testing. However, an additional six substances that did not produce positive results in the human testing were positive in the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5/12), a sensitivity of 75% (3/4), a specificity of 25% (2/8), a false positive rate of 75% (6/8) and a false negative rate of 25% (1/4). There are no comparative data from guinea pig tests with these fragrance ingredients. Therefore, a comparison of the performance of the LLNA and the guinea pig tests relative to the human outcome is not possible.

Use of the LLNA for Testing Metal Compounds

The evaluation of LLNA results for testing metal compounds has not changed from that in the January 2008 draft addendum. The NICEATM LLNA database contains test results on 48 studies involving 17 metal compounds representing 13 different metals (mixtures containing metals are excluded from this analysis). 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 nonsensitizer 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 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 83% (5/6), a false positive rate of 100% (1/1) and a false negative rate of 0% (0/5). By comparison, the guinea pig test had an accuracy of 100% (6/6), a false positive rate of 0% (0/1) and a false negative rate of 0% (0/5) against the human.

Use of the LLNA for Substances Tested in Aqueous Solutions

The evaluation of the LLNA for substances tested in aqueous solutions includes 118 additional substances compared with that of the January 2008 draft addendum. The revised NICEATM LLNA database for aqueous solutions contains test data on 171 studies that involved testing 139 substances; 91 (123 LLNA studies) of these substances are pesticide formulations and pure compounds, and 48 of these substances (48 LLNA studies) are aqueous eluates of medical devices. Because of differences in the protocols for sample preparation between the 91 pesticide formulations and pure compounds and the 48 medical device eluates, these groups were analyzed separately. Of the 91 pesticide formulations and pure compounds, 63% (57/91) are LLNA positive and 37% (34/91) are LLNA negative. LLNA studies were done with either CBA (66 studies) and/or BALBc (28 studies) mouse strains. The mouse strain was unspecified for 29 studies. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water.

Guinea pig data were available for 24 (4 sensitizers/20 nonsensitizers in the guinea pig) substances tested in aqueous solutions. Eleven substances were discordant between the

LLNA and the guinea pig tests. Ten of the 11 discordant substances were pesticide formulations tested in aqueous 1% Pluronic L92; these were the same 10 substances previously discussed for the pesticide formulations analysis, and all were overpredicted by the LLNA with respect to the guinea pig results (50% [10/20] overprediction). One additional substance, neomycin sulfate, which was tested in 25% EtOH, was underpredicted by the LLNA with respect to the guinea pig results (25% [1/4] underprediction). Overall, the LLNA and the guinea pig results were in agreement 54% (13/24) of the time.

Human data were available for only four substances (3 sensitizers/1 nonsensitizer in humans) tested in aqueous solutions, while there were only two substances tested in aqueous solutions in the LLNA for which there was comparative guinea pig and human data. Therefore the database of substances tested in multiple test methods (i.e., LLNA, guinea pig, and/or human) is too few to allow for a meaningful calculation.

All 48 of the medical device eluates were negative in the LLNA. None of these eluates had associated guinea pig or human data. These eluates were not analyzed to determine their constituents, or whether in fact any compound(s) were eluted from the medical device tested. Since the LLNA results were uniformly negative and no sample preparation control was included in the studies, the effectiveness of the sample preparation could not be determined. Therefore, the results from these eluates were not included with those from the pesticide formulations and pure substances tested in aqueous solutions.

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1.0 Introduction

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin-sensitizing chemicals and products. ACD results in lost workdays and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). To minimize the occurrence of ACD, regulatory authorities require testing to identify substances that may cause ACD. Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary to avoid development of ACD.

Skin sensitization testing has typically required the use of guinea pigs (Buehler 1965; Magnusson and Kligman 1970). However, in 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999). ICCVAM concluded that the LLNA (referred to herein as the "traditional LLNA") provided several advantages compared to the guinea pig method, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for ACD testing. It is now commonly used around the world.

In February 1998, 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 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 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 (GP) 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[6]).

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 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 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.

[6] 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).

Environmental Protection Agency [EPA] Health Effect Testing Guidelines on Skin Sensitization [EPA 2003]).

NICEATM conducted this revised evaluation of the LLNA applicability domain in response to a nomination[7] submitted to ICCVAM in January 2007 by the U.S. Consumer Product Safety Commission. This addendum to the ICCVAM (1999) report contains an evaluation of the current database for the LLNA when used to test pesticide formulations and other products, metals, and substances in aqueous solutions in order to fill some of the data gaps identified in the original evaluation (see **Appendix A**).

An independent peer review panel (Panel) reviewed this addendum in March 2008 to evaluate the extent to which the information contained in this addendum supported the draft recommendations. The draft recommendations stated that more data would be needed before a recommendation on the usefulness and limitations of the traditional LLNA for testing mixtures could be made, due to the limitations associated with the available mixtures database (i.e., unknown formulae, lack of human data). The Panel agreed that the draft recommendation with respect to the traditional LLNA testing of mixtures appeared valid based on the limitations inherent in the available data set. Still, the Panel urged that the ICCVAM recommendations indicate that the approach may be viable. The Panel further recommended that the test method recommendations summary should indicate that the limitations include relatively poor concordance of traditional LLNA outcomes for mixtures with to those obtained in GP tests. Routine comparisons of accuracy according to classification criteria may not be sufficient to evaluate the concordance for mixtures, and furthermore, the GP tests are not necessarily valid for mixtures. The Panel also indicated that the term *mixtures* was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations of mixtures that are being examined. The analyses in this addendum have been done separately on pesticide formulations, dyes, and fragrance ingredients in response to the Panel's comment.

The draft recommendations also stated that, based on the available data for metals, the traditional LLNA was useful for the testing of metal compounds, with the exception of nickel. Based on the available information, the Panel agreed that the draft recommendations

with regard to testing metals appeared to be valid. A minority Panel opinion stated that it should not be concluded that the traditional LLNA was not suitable for testing nickel compounds, because the different vehicles used may have had a significant impact on the ability of nickel to penetrate the skin and be bioavailable.

The draft recommendations also stated that, due to the limited number of substances tested in aqueous solutions, more data would be needed before a recommendation on the usefulness and limitations of the traditional LLNA for testing substances in aqueous solutions could be made. The Panel agreed that the draft ICCVAM recommendation was appropriate and that more data were required before an adequate evaluation of the use of the traditional LLNA with aqueous solutions could be conducted.[8]

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[9]). 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 (mixture* OR formula*)" OR ("metal* OR aqueous*)". Additionally, a weekly search on SCOPUS that uses the key words (TITLE-ABS-KEY(**sensi***) AND TITLE-ABS-KEY(**skin** OR **dermal**)) is done. Since March 2008, six relevant papers were added to the database.

[7] available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[8] available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[9] available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

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

This section reflects substances subsequent to the release of the draft addendum. These are summarized as follows:

- LLNA data and corresponding *in vivo* guinea pig test method data for 52 pesticide formulations were submitted by Dow AgroSciences.
- LLNA data for 28 pesticide formulations were submitted by Dupont Chemical Company.
- Detailed LLNA study results and corresponding human data for 12 fragrance ingredients were submitted by the Research Institute for Fragrance Materials. The summary results were originally published in Lalko and Api (2006).
- LLNA data for 48 medical device eluates were submitted by AppTec Laboratory Services.

These new data sources have been added to **Table 2.1**.

The information summarized in this addendum is based on a retrospective review of LLNA data derived from a database of over 600 substances (including pesticide formulations and other products) 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 formulations are not included in the analysis of pesticide formulations and other products, and metal compounds were restricted to those testing single substances. 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 pesticide formulations and other products, 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

584 response to the *FR* notice. **Table 2-1** provides information on the sources of the data and the
585 rationale for the substances tested.

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

Data Source	N	Substance Selection Rationale
AppTec Laboratory Services	48	Aqueous eluates from medical devices.
Dow AgroSciences	52	Pesticide formulations analyzed in the LLNA with associated GP data of various kinds.
Dupont	28	Pesticide formulations analyzed in the LLNA
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, 1999a, 2005)	16	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Lalko and Api (2006)	12	Original research that evaluated essential oils in the LLNA. Additional data were submitted by the authors and RIFM.
Ryan et al. (2000)	2	Interlaboratory study to evaluate the accuracy of the LLNA to identify human sensitizers.
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	6	Original LLNA research on 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	**262**	

Abbreviations: BGIA = Berufsgenossenschaftliches Institut fur Arbeitsschutz; CESIO = Comite Europeen des Agents de Surface et de Leurs Intermediaires Organiques; CPSC = U.S. Consumer Product Safety Commission; ECPA = European Crop Protection Association; GP = guinea pig; 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; NTP = National Toxicology Program; RIFM = Research Institute for Fragrance Materials: 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.

LLNA studies for 29/89 of the pesticide formulations (tested in aqueous solutions) used the BALB/c mouse strain rather than the CBA/J and CBA/Ca strains of mice, which are recommended for the LLNA by ICCVAM (ICCVAM 1999, Dean et al. 2001, EPA 2003), and the OECD (OECD 2002). The comparative performance of the LLNA using these different strains relative to the guinea pig is detailed in **Section 5.0**. Two additional submitted LLNA studies (from Dr. Dori Germolec at the National Institute of Environmental Health Sciences [NIEHS]) also used the BALB/c strain. 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.

To the extent possible, **Appendices B1**, **B4**, **B6**, **C1**, and **D1** provide information on the physico-chemical properties (e.g., physical form), Chemical Abstracts Service Registry Number (CASRN), and chemical class for each pesticide formulation, dye, fragrance ingredient, metal compound, and substance tested in an aqueous solution, respectively. This information was obtained from published reports, submitted data, or through literature searches.

When available, chemical classes for the test substances were retrieved from the National Library of Medicine's ChemID Plus database. If chemical classes were not located, where possible, 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[10]. 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. Material families for the active ingredients in the formulations submitted by Dow AgroSciences were provided by Dow AgroSciences.

The generic composition of some of the formulated products evaluated by the European Crop Protection Association (ECPA) (Dinocap EC, Oxyflourfen EC, Quinoxyfen/cyproconazole, and Trifluralin EC) and the formulations submitted by Dow AgroSciences, using the LLNA, is included in **Appendix B3**. For the formulations provided by ECPA, none of the active ingredients have been tested using the LLNA but the active ingredients have been tested previously in a guinea pig test (personal communication by Dr Eric Debruyne, Bayer CropScience in France). Likewise, none of the inerts (e.g., surfactants, solvents, etc.) have been tested independently for these formulations. Dow AgroSciences provided information about LLNA and guinea pig test on active ingredients and inerts for the formulations they submitted. The component information for the remaining pesticide formulations have been requested by NICEATM, but since some of the data is proprietary, it is not available at this time.

One hundred and four pesticide formulations (i.e., herbicides, fungicides, insecticides) were evaluated for this addendum. All of these were liquids, though some were in the form of suspensions or emulsions, and were tested in an aqueous vehicle. Six dyes (all solids), and 12 fragrance ingredients (all liquids), which are a combination of essential oils and absolutes, were also evaluated. Essential oils are oils derived from a natural source using steam or pressure. Absolutes are purified extracts from natural products. Both essential oils and absolutes are substances comprised of more than one component.

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 physical form is identified are solids.

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

649 Of the 21 substances tested in aqueous solutions included in this evaluation, six are pesticides
650 (i.e., herbicide, fungicides, and insecticides); this is the only product class represented by
651 more than one substance tested in an aqueous solution.

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3.0 Comparative *In Vivo* Reference Data

The *in vivo* reference data in this draft addendum has been revised from the January 2008 draft addendum to include data received subsequent to the release of the draft addendum. These data are summarized in **Section 2.0**. 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 2007). 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), and Dow AgroSciences, were reportedly conducted according to GLP guidelines. None of the published references from which GP or human data were obtained include specifics on GCP or GLP compliance.

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4.0 LLNA Data and Results

The test method data in this draft addendum has been revised from the January 2008 draft addendum to include data received subsequent to the release of the draft addendum. These data are summarized in **Section 2.0**. The data used for this evaluation were obtained from 25 sources (**Table 2-1**). No new LLNA studies were conducted to generate data for this evaluation (see **Section 2.0**). Where available, specific information including name, CASRN, physico-chemical properties (e.g., molecular weight, Log K_{ow}), chemical class[11] and data source are indicated for each pesticide formulation, dye, fragrance ingredient, metal compound, and substance tested in an aqueous solution (**Appendices B1, B4, B6, C1,** and **D1**, respectively). The concentrations tested, along with calculated stimulation index (SI) and/or EC3 (the concentration that induces an SI of 3) values, are provided in **Appendices B2**, **B5, B7, C2,** and **D2** for pesticide formulations, dyes, fragrance ingredients, metal compounds, and substances tested in an aqueous solution, respectively. Individual components and concentrations of the pesticide formulations and substances tested in an aqueous solution submitted by Bayer have been requested, but due to confidential and proprietary issues, Bayer has only been able to provide the generic composition for four formulated products (see **Section 2.0**). Furthermore, provided in the submitted data or study reports, the source or purity of the test substance was not known.

LLNA classification as to whether a substance was a sensitizer or a nonsensitizer was based on study data extracted from the sources listed in **Table 2-1** and **Appendices B1, B4, B6, 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. 1999a) 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

741 previous evaluation of 209 substances (ICCVAM 1999; **Appendix A**) or for any of the newly
742 obtained studies used in this evaluation.

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[11] 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).

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5.0 Accuracy of the LLNA: Revised Applicability Domain

Since the publication of the draft addendum in 2008, NICEATM obtained additional LLNA data, which were used to revise the evaluation of the LLNA for testing pesticide formulations and other products[12](**Section 5.1**) and for testing substances in aqueous solutions (**Section 5.3**). No new LLNA data were received for LLNA tests with metals, therefore this evaluation remains unchanged (**Section 5.2**). The new data contained in this revised addendum are summarized in **Section 2.0.**

The ability of the LLNA to correctly identify pesticide formulations and other products, 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 pesticide formulations, dyes, fragrance ingredients, metal compounds, and substances tested in aqueous solutions and the relevant data for each substance is located in **Appendices B2**, **B5, B7, 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 5-3**, **5-6**, **5-8**, **5-10**, and **5-13**. For this addendum, substances containing multiple components were analyzed separately as pesticide formulations, dyes, and fragrance ingredients.

5.1 Testing of Pesticide Formulations and Other Products

The original ICCVAM LLNA report (ICCVAM 1999) (**Appendix A**) did not include an analysis on the ability of the LLNA to predict the skin sensitizing potential of pesticide formulations and other products, because data were not available for that evaluation. Thus, all of the analyses below for pesticide formulations, dyes and fragrance ingredients are new material in this addendum.

5.1.1 *Testing of Pesticide Formulations*

The current LLNA database contains data for 104 pesticide formulations for which LLNA data exists. The physico-chemical properties of these formulations are in **Appendix B1,** and the data analyzed here are in **Appendix B2**.

For these formulations, 54% (56/104) were classified as sensitizers in the LLNA, and 46% (48/104) were classified as nonsensitizers. For substances that were tested multiple times in the LLNA, classification as a sensitizer or nonsensitizer was made by a majority call; i.e., the most prevalent call that occurred among the studies considered. For example, five independent studies were considered for the formulation Oxyfluorfen EC. The highest SI values observed for the various studies were 5.4, 4.9, 3.1, 2.8, and 2.3, respectively (all of these SI values occurred with a test concentration of 33%). Since an SI value ≥ 3 occurred in three of the five studies, Oxyfluorfen EC was classified as a sensitizer in the LLNA, even though two studies (SIs = 2.8 and 2.1, respectively) would have resulted in classification as a nonsensitizer if considered alone.

Seventy of the 104 pesticide formulations have LLNA and some type of guinea pig reference data. A total of 89 LLNA studies were performed using these 70 formulations. LLNA studies were conducted with either CBA/Ca or CBA/J (61/89) and/or BALBc (28/89) mouse strains.

Six formulations were tested in multiple LLNA studies (25 studies total [**Table 5-1]**). LLNA results for 5/6 formulations were in agreement across multiple studies, and LLNA results for 1/6 formulations were discordant across multiple studies (3 positive, 2 negative [**Table 5-2**]).

Twenty-two formulations had associated GP data for the formulation itself, 46 formulations had GP data for one or more of the active ingredients in the formulation, and 14 formulations had GP data for a substance related to an active ingredient, or for a related formulation. The performance of the LLNA against GP tests for pesticide formulations with GP data for the entire formulation is discussed in **Section 5.1.1.1**, below. The performance of the LLNA against GP tests for pesticide formulations with GP data for active ingredients or related substances and formulations is discussed in **Appendix E.**

All formulations (89/89 studies) were tested in the LLNA in 1% Pluronic L92. Pluronic L92 block copolymer is a surfactant and wetting agent that has been evaluated as an alternative aqueous-based vehicle for use in the LLNA. Pluronic L92 was chosen for evaluation because it promotes test material retention on the ear by preventing run-off, and exhibits low acute toxicity and irritation potential (Boverhof et al. 2008; Ryan et al. 2002). Ryan et al. (2002)

[12] Based on the Panel recommendation, this revised addendum does not refer to "mixtures" as a type of

assessed the performance of Pluronic L92 relative to other solvents in the LLNA using aqueous soluble haptens. Based on their results, they determined that, for identification of sensitization hazard of aqueous soluble materials using the LLNA, dimethylformamide (DMF), and dimethylsulfoxide (DMSO) were the preferred vehicles. However, if a test material is not soluble in DMF or DMSO, or if higher test concentrations could be achieved in an aqueous vehicle, then 1% Pluronic L92 might improve assay performance over the use of water as a vehicle.

In an inter-laboratory study (n=5 laboratories), Boverhof et al. (2008) conducted LLNA tests on three substances with known sensitization potential and four pesticide formulations for which the sensitization potential in guinea pigs and/or humans had previously been determined, along with three commonly-used positive controls in sensitization testing (hexylcinnamaldehyde, formaldehyde, and potassium dichromate), using Pluronic L92 as the vehicle. They concluded that the LLNA results for all of these substances when tested in Pluronic L92 were consistent with previous GP or human results, and that Pluronic L92 was a suitable vehicle to use when testing aqueous solutions in the LLNA.

For the 52 formulations submitted by Dow AgroSciences, a list of all of the components in the formulation (albeit some were listed generically [e.g., emulsifier, biocide, etc.]) was also provided, along with information as to whether each component was a sensitizer). For these components, the criteria for classification as a sensitizer were not specified. **Appendix B3** contains the information on components provided by Dow AgroSciences.

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substance tested, but rather specifies the types of products that were tested, where possible.

Table 5-1 Pesticide Formulations with Multiple LLNA Studies

Formulation	Source	No. Studies	Mouse Strain	No. Positive Studies	No. Negative Studies	No. of Labs
Atrazine SC	ECPA	2	CBA	2	0	2
Dinocap EC	ECPA	5	CBA	5	0	5
Formulation 7	Dow AgroSciences	2	BALB/c	2	0	1
Oxyflouren EC	ECPA	5	CBA	3	2	5
Quinoxyfen/cyproconazole	ECPA	6	CBA	6	0	6
Trifluralin EC	ECPA	5	CBA	5	0	5

Abbreviations: EC = emulsion concentrate; ECPA= European Crop Protection Association; No. = Number; SC = suspension concentrate;

Table 5-2 LLNA Data for Pesticide Formulation with Discordant Results

Formulation	Vehicle	Conc. (%)	SIs	Strain	EC3 (%)	Lab
Oxyfluorfen EC	L92	1, 7, 33	0.8, 1.4, 4.9	CBA/Ca	30.8	1
		1, 7, 33	0.9, 1.4, 2.8	CBA/J	NC	2
		1, 7, 33	0.3, 0.9, 2.3	CBA/J	NC	3
		1, 7, 33	1.1, 1.5, 3.1	CBA/JHsd	30.8	4
		1, 7, 33	1.2, 1.2, 5.4	CBA/CaOlaHsd	18.1	5

Abbreviations: Conc. = Concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce an SI of 3; L92 = 1% aqueous pluronic L92; NC = Not calculated since SI<3.0; SIs = Stimulation indices

5.1.1.1 *Testing of Pesticide Formulations: LLNA vs. GP with Available Reference Data for the Entire Formulation*

For the 22 formulations that had associated GP data for the formulation itself, 14% (3/22) were classified as sensitizers and 86% (19/22) as nonsensitizers according to the GP results (**Figure 5-1**). Twenty of these GP tests were BT and 2 were GPMT. These results are based on a positive overall GP call for formulation EXP 10810[13]. Nine out of the approximately 450 active ingredients registered with EPA were represented among these 22 formulations. Furthermore, approximately 40 different classes of pesticides are registered with EPA, of which these nine active ingredients represent a small proportion (i.e., one insecticide, six herbicides and two fungicides).

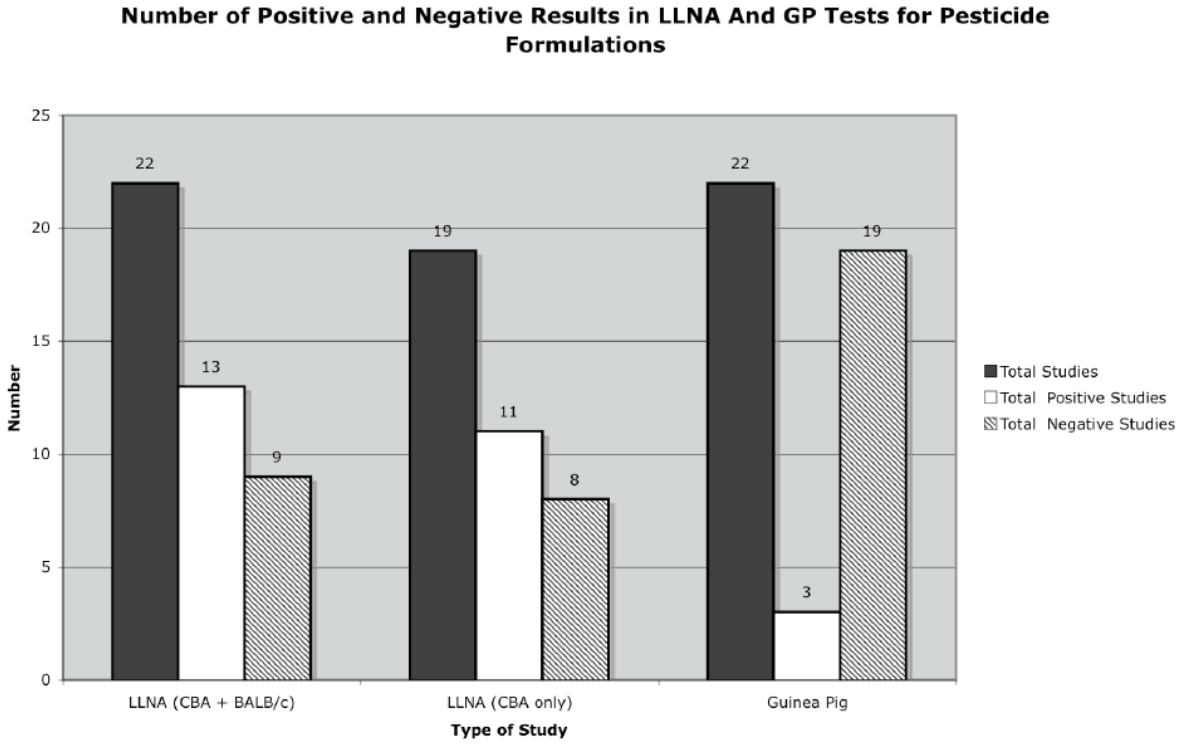
Nineteen of the LLNA studies were conducted in CBA mice (i.e., the preferred strain for use in the LLNA according to the ICCVAM recommended LLNA protocol and OECD TG 429) and three studies were conducted in BALB/c mice. The LLNA classified 59% (13/22) of the formulations as sensitizers and 41% (9/22) as nonsensitizers (**Figure 5-1**). All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA. The LLNA also identified an additional seven substances as sensitizers that were classified as nonsensitizers in the GP test (**Table 5-3**).

If only LLNA studies using CBA mice are considered, three LLNA studies conducted with BALB/c mice are removed from the database, which eliminates two LLNA positive studies, and one LLNA negative study. Based on the remaining 19 LLNA studies, the LLNA classified 58% (11/19) of the formulations as sensitizers and 42% (8/19) as nonsensitizers (**Figure 5-1**). This does not change the fact that all three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA, and that seven substances identified as sensitizers in the LLNA are classified as nonsensitizers in the GP test (**Table 5-3**).

[13] Formulation EXP 10810 A (submitted by E. Debruyne, Bayer Crop Science), the only formulation for which there was data in both the GPMT and the BT, showed equivocal results in the guinea pig. This formulation tested positive in the GPMT (sensitization incidence 100%), and negative in the BT (sensitization incidence 10%). The patch concentration in the GPMT was the same as the induction concentration in the BT (50%).

There were no comparative human data with which to determine the actual human sensitization potential.

Figure 5-1 Numbers of Positive and Negative LLNA and GP Calls for Pesticide Formulations



Abbreviations: BALB/c = LLNA studies conducted using the BALB/c mouse strain; CBA = LLNA studies conducted using the CBA mouse strain; GP = guinea pig; LLNA = local lymph node assay

Based on the 22 pesticide formulations tested in CBA (n=19) and BALBc (n=3) strains, the accuracy of the LLNA compared to guinea pig data was 54% (12/22), the sensitivity was 100% (3/3), the specificity was 47% (9/19), the false positive rate was 53% (10/19) and false negative rate was 0% (0/3). If the three studies using BALB/c mice are not considered, the accuracy of the LLNA compared to guinea pig data was 58% (11/19), the sensitivity was 100% (3/3), the specificity was 50% (8/16), the false positive rate was 50% (8/19) and false negative rate was 0% (0/3) (**Table 5-3**).

Table 5-3 Evaluation of the Performance of the LLNA for Testing Pesticide Formulations

Comparison[1]	n[2]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]
LLNA[4] vs. GP[5] (Formulation[6])	22	54	12/22	100	3/3	47	9/19	53	10/19	0	0/3
LLNA[7] vs. GP[5] (Formulation[6])	19	58	11/19	100	3/3	50	8/16	50	8/16	0	0/3
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[8]											
LLNA[7] vs. GP[5]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA[7] vs. Human[9]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[5] vs. Human[9]	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 formulations that have LLNA data and some type of associated GP data; none of the pesticide formulations 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] LLNA studies conducted with CBA (n=19) and BALBc (n=3) mice.

[5] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[6] Formulation refers to associated GP data for the formulation itself.

[7] LLNA studies conducted with CBA mice.

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

[9] 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.

Among the 10 of 22 formulations classified as sensitizers by the LLNA that were classified as nonsensitizers in the GP (**Table 5-4**), eight were classified as nonsensitizers based on BT results and two were classified as nonsensitizers based on GPMT results.

Table 5-4 Pesticide Formulations that are Classified as Sensitizers in the LLNA, but Classified as Nonsensitizers in the GP

Substance Name	LLNA Results				GP Results			Skin Irritant?
	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Ind. Conc. (%)	Sens. Incid. (%)	Result[3]	
Atrazine SC	100	7.3	36.4[4]	+	30	0	-[5]	Nonirritant at ≤ 25%[6]
BASF SE-1	70	22.7	5.5	+	100	0	-[7]	Nonirritant at ≤ 50%[6]
EXP 11120 A	100	5.3	64.9	+	100	0	-[7]	Nonirritant at 100%[6]
F & Fo WG 50 + 25	25	15.2	0.003	+	30	0	-[7]	Nonirritant at ≤ 10%[6]
FAR01060-00	100	3.6	88.5	+	100	0	-[7]	Nonirritant at 100%[6]
Formulation 2[8]	80	15.8	15.7	+	NA	NA	-[7]	Nonirritant at 80%[9]
Formulation 7[8]	100	3.2	85	+	100	0	-[7]	Nonirritant at 80%[9]
Fx + Me EW 69	50	8.6	25.2	+	100	0	-[7]	Nonirritant at 100%[6]
Oxyfluorfen EC	33	5.4	30.8[10]	+	10	26	-[5]	Nonirritant at ≤ 25%[6]
Trifluralin EC	100	75.2	10.3[11]	+	50	10	-[7]	Nonirritant at ≤ 25%[6]

Abbreviations: Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of 3; EW = emulsion, oil in water; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; SC = suspension concentrate; Sens. Incid. = sensitization incidence; SI = stimulation index; WG = water-dispersible granules

[1]Maximum concentration tested in the LLNA

[2]Maximum SI obtained in the LLNA

[3] (-) = nonsensitizer, (+) = sensitizer

[4]Mean value from 2 studies

[5]Guinea pig maximization test (GPMT) result

[6]Based on challenge concentration from a GPMT or Buehler test (BT

[7])BT result

[8]LLNA conducted in BALB/c mice

[9]Based on irritation prescreen in mice

[10]Mean from 3 positive studies

[11]Mean of 5 studies

The constituents of most of the formulations are unknown (**Appendix B3**). Formulation 2 contains a biocide (at a concentration of 0.54 g/L) that is a sensitizer according to constituent information provided by Dow AgroSciences (**Appendix B3**). Dow Agrosciences categorizes all other constituents of Formulation 2 as nonsensitizers, including the active ingredients Fluroxypyr-meptyl and Florasulam (**Appendix B3**). Formulation 7 contains the sensitizers quinoxyfen (active ingredient at a concentration of 45 g/L) and a biocide (at a concentration of 0.37 g/L); it is unknown whether this is the same biocide that is a constituent of Formulation 2. Formulation 7 also contains the active ingredient mycyclobutanil, which, when tested by Dow AgroSciences in GP sensitization tests, gave equivocal results (**Appendix B3**).

Six of the overpredicted formulations based on LLNA results compared to GP results (BASF SE-1, EXP 11120 A, F & Fo WG 50 + 25, FAR01060-00, Formulation 7, and Fx + Me EW 69; see **Table 5-4**) were tested in the GP at induction concentrations equal to or greater than the highest concentration tested in the LLNA. However, atrazine tested as a sensitizer at 100% in the LLNA, but tested as a nonsensitizer at 30% induction concentration in the GPMT; oxyflourfen tested as a sensitizer at 33% in the LLNA, but tested as a nonsensitizer at 10% induction concentration in the GPMT; and trifluralin tested as a sensitizer at 100% in the LLNA, but tested as a nonsensitizer at 50% induction concentration in the BT **(Table 5-4**).

The EC3 values for most (9/10) of the formulations indicated that they produced weak to moderate responses in the LLNA (EC3 range of 5.5% to 88.5%) (**Table 5-4**). However, the EC3 value for the formulation F & Fo WG 50 + 25 (EC3 = 0.003%) is a very strong LLNA response. This could be due to the observed SI values on the LLNA dose-response curve that were used to calculate an EC3 by extrapolation (because no points fall below SI = 3) approach saturation (SI = 11.7 at 2.5%, SI = 15.2 at 25%) (**Appendix B2**). This EC3 value is likely a poor estimate of the actual value. However, based on the concentrations test, and the resulting SI values, the LLNA data do indicate that the EC3 for formulation F & Fo WG 50 + 25 is less than 2.5% (i.e., SI = 11.7 at 2.5%, the lowest concentration tested).

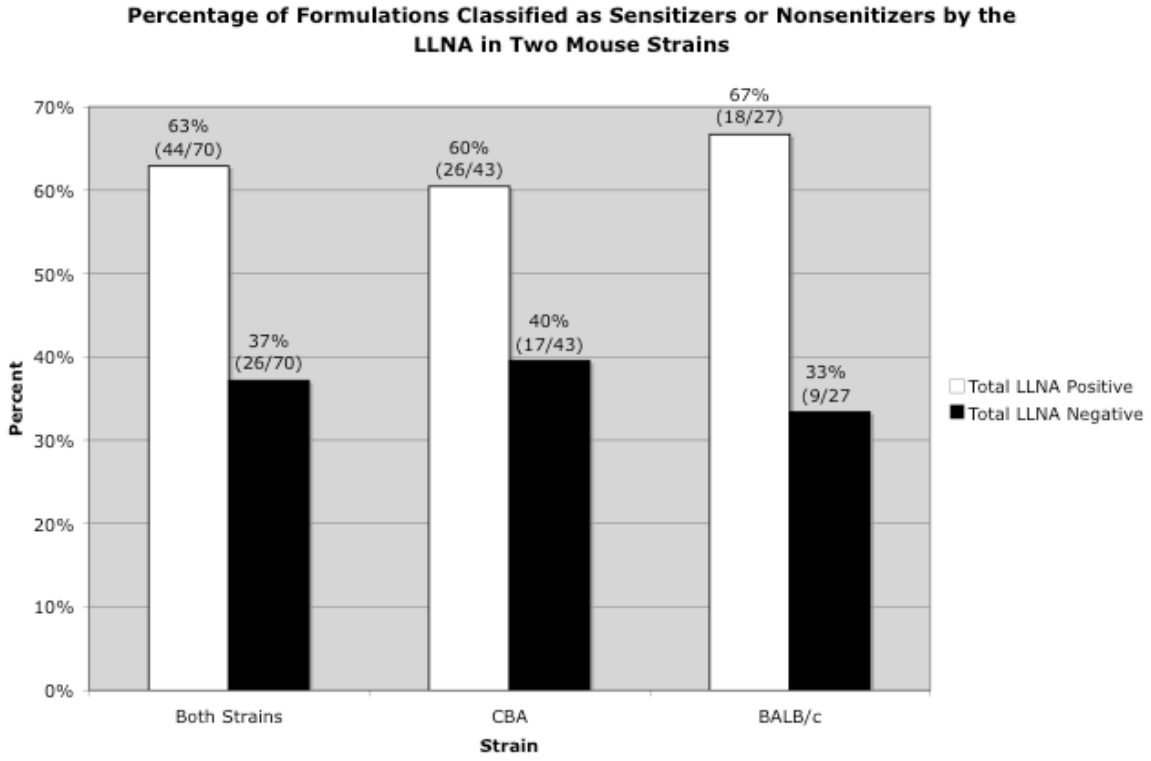
Five of the overpredicted formulations (Atrazine SC, BASF SE-1, F & Fo WG 50 + 25, Oxyflourfen EC and Trifluralin EC) were tested in the LLNA at potentially irritating concentrations. This is based on the concentration tested in the LLNA exceeding the reported challenge concentrations used in the BT or GPMT. According to the respective protocols for these guinea pig tests, the challenge concentration should be the maximum nonirritating concentration of a test substance (**Table 5-4**).

5.1.1.2 *Testing of Pesticide Formulations: Comparison Between Mouse Strains CBA and BALB/c*

For the 70 pesticide formulations that had associated GP data, 43 were tested in the LLNA in CBA mice and 27 were tested in BALB/c mice. No formulation was tested in the LLNA in both strains. **Figure 5-2** shows that the percentage of formulations that were classified as

958 sensitizers was slightly higher in BALB/c mice (67% [18/27]) than in CBA mice (60%
959 [26/43]).

960 **Figure 5-2 Percentage of Formulations Classified as Sensitizers or Nonsensitizers in**
961 **Two Mouse Strains**



962
963 For the 22 pesticide formulations that were tested in the GP as entire formulations, the LLNA
964 studies for 19/22 were conducted using CBA mice and 3/22 were conducted using BALBc
965 mice. As noted in **Section 5.1.1.1, w**hen data for all 22 formulations is considered (i.e., using
966 both CBA and BALB/c data), the overall accuracy is 54% (12/22), with false positive and
967 false negative rates of 53% (10/19) and 0% (0/3), respectively. If only LLNA studies using
968 CBA mice are considered, removing the three LLNA studies conducted with BALB/c mice
969 from the database eliminates two LLNA positive studies, and one LLNA negative study,
970 which only marginally impacts the overall accuracy (accuracy = 58% [11/19], false positive
971 rate = 50% [8/16], and false negative rate = 0% [0/3]).

972 As mentioned previously, since comparative human data are not available for any of the
973 formulations analyzed, an evaluation of these formulations in the LLNA compared to human

performance could not be assessed. For the same reason, an evaluation of GP versus human outcomes is also not possible. Also, no formulations were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**), so these data and analyses cannot be compared to previously considered data.

5.1.2 *Testing of Dyes*

The current LLNA database contains data for six dyes, for which there is LLNA and GP data. The physico-chemical properties of these dyes are in **Appendix B4,** and the data analyzed here are in **Appendix B5**. For these dyes, 50% (3/6) were classified as sensitizers in the LLNA, and 50% (3/6) were classified as nonsensitizers in the LLNA. In the GPMT, 83% (5/6) dyes tested as sensitizers. **Table 5-5** provides the performance statistics for the LLNA when compared to GPMT outcomes for this limited dataset.

Table 5-5 Evaluation of the Performance of the LLNA for Testing Dyes

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. GPMT	6	33	2/6	40	2/5	0	0/1	100	1/1	60	3/5
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[4]											
LLNA vs. GP[5]	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[5] vs. Human[6]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations: GP = guinea pig; GPMT = guinea pig maximization test; 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 dyes that have LLNA data and some type of associated GP data; none of the dyes 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] For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**) showing the overall performance of the LLNA vs. GP and human, and GP versus human is included here.

[5] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test, the Buehler test, or the McGuire test.

[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.

Four of the six dyes showed discordant results between the LLNA and the GPMT. These substances are shown in **Table 5-6**, including the maximum concentration tested in the LLNA and the maximum SI value attained, as well as the induction concentration and sensitization incidence in the GPMT. These results indicate that the discordant outcomes between the LLNA and the GPMT cannot be explained based on the concentrations tested

(i.e., the maximum concentration tested in the LLNA was higher than the GPMT induction concentration in all four cases).

Table 5-6 Dyes Discordant Between the LLNA and GPMT

Substance Name	LLNA Results					GPMT Results			Skin Irritant?
	Veh.	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Ind. Conc. (%)	Sens. Incid. (%)	Result[3]	
C.I. Reactive Yellow 174	AOO	15	7.8	7.8	+	5	11	-	NA
Dispersionsrot 2754	AOO	9	1	NC	-	5	100	+	NA
Produkt P-4G	AOO	15	2.5	NC	-	5	90	+	NA
Yellow E-JD 3442	AOO	15	0.9	NC	-	5	90	+	NA

Abbreviations: AOO = acetone/olive oil; Conc. = concentration; EC3 = estimated concentration needed to produce a stimulation index of three; GPMT = guinea pig maximization test; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI<3.0; ND = not done; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle

[1]Maximum concentration tested in the LLNA.

[2]Maximum SI obtained in the LLNA.

[3] (-) = nonsensitizer, (+) = sensitizer

As mentioned previously, since comparative human data are not available for any of the dyes analyzed, an evaluation of these substances in the LLNA or the GP compared to human performance could not be assessed. Also, no dyes were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**), so these data and analyses cannot be compared to previously considered data.

5.1.3 *Testing of Fragrance Ingredients*

The current LLNA database contains data for 12 fragrance ingredients, for which there are LLNA and human data. The physico-chemical properties of these fragrance ingredients are in **Appendix B6,** and the data analyzed here are in **Appendix B7**. For these fragrance ingredients, 75% (9/12) were classified as sensitizers in the LLNA, and 25% (3/12) were classified as nonsensitizers in the LLNA. In the human, 33% (4/12) of these substances tested as sensitizers. One of these human sensitizers (treemoss) was underpredicted by the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5/12), a sensitivity of 75% (3/4), a specificity of 25% (2/8), a false positive rate of 75% (6/8) and a false negative rate of 25% (1/4) (**Table 5-7**).

Table 5-7 Evaluation of the Performance of the LLNA for Testing Fragrance Ingredients

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. Human[4]	12	42	5/12	75	3/4	25	2/8	75	6/8	25	1/4
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[6]											
LLNA vs. GP[5]	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; 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 substances that have LLNA data and associated human data; none of the fragrance ingredients analyzed had GP 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] 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] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test, the Buehler test, or the McGuire test.

Seven of 12 fragrance ingredients showed discordant results between the LLNA and the HMT. These substances are shown in **Table 5-8**, along with the maximum concentration tested in the LLNA and the maximum SI value attained, and the test concentration and sensitization incidence from the HMT. Most (6/7) of the discordant substances were LLNA positive/human negative. All substances for which concentration information was available for both the LLNA and HMT (5/7) were tested at higher concentrations in the LLNA than the induction concentration in the HMT. All false positives in the LLNA produced maximum SI values greater than 6.0, with the exception of spearmint oil, which produced an SI of 3.6 at a test concentration of 10%. All of the discordant LLNA positive fragrance ingredients had EC3 values in a narrow range (3.6% to 9.6%). All false positives were clearly nonsensitizers in the HMT with a sensitization index of 0%. The one human sensitizer underpredicted by the LLNA (treemoss) is classified as a sensitizer based on a sensitization incidence of 2% (3/145) in humans. The concentrations tested in the LLNA and the human were not available.

Table 5-8 Fragrance Ingredients: Discordant Results Between the LLNA and Human

Substance Name	LLNA Results					HMT Results			Skin Irritant?
	Veh.	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Test Conc. (%)	Sens. Incid. (%)	Result[3]	
Basil oil	EtOH/DEP (1:3)	50	25.2	6.2	+	4	0	-	Mild irritant at 100%[4]
Clove oil	EtOH/DEP (1:3)	50	11.4	7.1	+	5[5] 5[6] 10[7]	0[5] 0[6] 0[7]	-	Severe irritant at 100%[8]
Lemongrass oil	EtOH/DEP (1:3)	50	13.1	6.5	+	4[9] 4[10] 5[10]	0[9] 0[10] 0[10]	-	Mild irritant at 100%[4]
Litsea cubeb oil	EtOH/DEP (1:3)	50	16.0	8.4	+	8	0	-	Strong irritant at 100%[4]
Palmarosa oil	EtOH/DEP (1:3)	50	5.0	9.6	+	NA	0	-	NA
Spearmint oil	EtOH/DEP (1:3)	10	3.6	3.6	+	4	0	-	Nonirritant at 100%[4]
Treemoss	EtOH/DEP (1:3)	NA	NA	NC	-	NA	2[11]	+	Nonirritant at 100%[4]

Abbreviations: Conc. = concentration; DEP = diethyl phthalate: EtOH = ethanol: HMT = human maximization test; LLNA = local lymph node assay; NA = Not available; NC = Not calculated since SI<3.0; Sens. Incid. = Sensitization incidence; SI = Stimulation index; Veh. = Vehicle

[1] Maximum concentration tested in the LLNA.
[2] Maximum SI obtained in the LLNA.
[3] (-) = nonsensitizer, (+) = sensitizer
[4] Test in mice.
[5] Test substance was clove bud oil. (Opdyke 1975a)
[6] Test substance was clove stem oil (Opdyke 1975b)
[7] Test substance was clove leaf oil Madagascar (Opdyke 1978)
[8] Test in mice with clove stem oil. (Opdyke 1976a)
[9] Test substance was lemongrass oil, East Indian (Opdyke 1976a)
[10] Test substance was lemongrass oil, East Indian (Opdyke 1976b)
[11] HMT or human repeat insult patch test data, submitted by the Research Institute for Fragrance Materials.

As mentioned previously, since comparative GP data are not available for any of the fragrance ingredients analyzed, an evaluation of these substances in the LLNA compared to GP performance could not be assessed. For the same reason, an evaluation of GP versus human outcomes is also not possible. Also, no fragrance ingredients 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 evaluation of the LLNA for testing metal compounds has not changed from that in the January 2008 draft addendum. 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 revised to include a total number of 17 metal compounds, representing 13 different metals, with corresponding human and/or GP data The physico-chemical properties of these metal compounds are in **Appendix C1,** and the data analyzed here are in **Appendix C2**. To reduce the complexity of the analysis, pesticide formulations and other products 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 pesticide formulations and other products (**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 GP 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 nonsensitizer 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 nonsensitizers 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 5-9**). For the six metal compounds (after excluding nickel compounds) with GP data (five sensitizers and one nonsensitizer in the GP), 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 GP test results (**Table 5-9**) (**Appendix C2**).

Furthermore, all six of the 14 metal compounds with GP data have human data for comparison and there is a chemical-by-chemical match in classification between the GP and human outcomes (**Table 5-9**). In contrast, the LLNA incorrectly identified the one human non-sensitizing metal compound 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 5-9**.

Table 5-9 Evaluation of the Performance of the LLNA for 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. GP and human, and GP versus human is included here.

Of the six metal compounds with GP 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 GP data that was tested in an aqueous vehicle, it was a sensitizer in the

LLNA and the LLNA correctly classified it compared to the GP data (**Table 5-9**). All of the five metal compounds with comparative GP data tested in a non-aqueous vehicle are also classified as sensitizing in the LLNA. Compared to GP data, the LLNA correctly classifies four of the five non-aqueous metal compounds. The accuracy statistics based on this limited dataset are also presented in **Table 5-9**.

Of the 14 metal compounds with human data, the vehicle is known for 12 of the 14 compounds. Eleven 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 human data that was tested in an aqueous vehicle, the LLNA correctly classified it as a sensitizer compared to the human data (**Table 5-9**). In contrast, of the 12 metal compounds with comparative human data tested in a non-aqueous vehicle, eight are classified as sensitizers and the remaining four are nonsensitizers in the LLNA. Compared to human data, the LLNA correctly classifies 11 of the 12 non-aqueous metal compounds. This results in an accuracy of 92% (11/12), a sensitivity of 100% (7/7), a specificity of 80% (4/5), a false positive rate of 20% (1/5) and a false negative rate of 0% (0/7) (**Table 5-9**).

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

For the purpose of this addendum, a case-by-case analysis was carried out to determine whether the overall LLNA classification for each metal compound is as a sensitizer or a nonsensitizer. In most cases, the majority result determined the overall LLNA skin sensitizing classification for each metal compound. In instances where there were an equal number of reports classifying the metal compound as sensitizing or non-sensitizing, the most severe classification was used. For instance, for zinc sulfate, LLNA data from two studies are considered in this evaluation report (ICCVAM 1999 [**Appendix A**] and Basketter et al. 1999a). Zinc sulfate is classified as a sensitizer in ICCVAM 1999 (neither the vehicle nor the

raw data were included) whereas Basketter et al. (1999a) classified zinc sulfate as a nonsensitizer 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**). The evaluation of the LLNA for substances tested in aqueous solutions in this revised addendum includes 118 additional substances compared with that of the January 2008 draft addendum.

The revised database contains LLNA data for 139 substances tested in aqueous solutions, representing 171 LLNA studies; 91 (123 LLNA studies) of these substances are pesticide formulations and pure compounds and 48 of these substances (48 LLNA studies) are aqueous eluates of medical devices. As mentioned previously in **Section 5.1.1**, all pesticide formulations were tested in the LLNA in 1% Pluronic L92. Because of differences in the protocols for sample preparation between the 91 pesticide formulations and pure compounds and the 48 medical device eluates, these groups were analyzed separately.

In this addendum, the ICCVAM 1999 report has been revised to include a total of 24 unique substances tested in aqueous solutions from 46 LLNA studies with corresponding human and/or GP data. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water. The group of substances analyzed for this section of the addendum does not include metal compounds tested in aqueous vehicles, which have instead been included in the analyses discussed in **Section 5.2**.

5.3.1 *Pesticide Formulations and Pure Compounds Tested in Aqueous Solutions*

Of the 91 pesticide formulations and pure compounds considered in this analysis, 63% (57/91) are LLNA positive and 37% (34/91) are LLNA negative. Where available, the physico-chemical properties of these substances are in **Appendix D1,** and the data analyzed here are in **Appendix D2**. If there were multiple LLNA studies for a substance, a majority call was used, so there was one LLNA call for each substance. Eleven substances were tested in multiple LLNA studies (43 total studies); 9/11 of these substances had concordant LLNA results among all studies, and 2/11 substances had discordant results among 2 or more studies (**Table 5-10**).

LLNA data for the two substances for which discordant LLNA study results occurred are shown in **Table 5-11**. The discordance for 1,4 dihydroquinone is likely due to differing concentration ranges between the two LLNA studies (i.e., only one study tested up to at least 5%, where a positive result was first noted). For Oxyfluoren EC, the range of EC3 values for the positive LLNA studies (> 20%) is associated with a weak response in the LLNA, where the greatest variability would be expected. Similarly, the SI values for the negative LLNA studies (2.3 and 2.8) are near the threshold for a positive response (i.e., SI=3), again where the greatest variability would be expected **(Table 5-11)**.

Table 5-10 Substances Tested in Aqueous Solutions in Multiple LLNA Studies

Formulation	Reference	No. Studies	Mouse Strain	Vehicle	No. Positive Studies	No. Negative Studies	No. of Labs
Atrazine SC	ECPA	2	CBA	L92	2	0	2
1,4 Dihydroquinone	Lea et al. (1999)	2	NA	ACE/saline (1:1)	1	1	2
2,4 Dinitrobenzene sulfonic acid	Ryan et al. (2002)	2	NA	L92	2	0	1
				H_2O			
Dinocap EC	ECPA	5	CBA	L92	5	0	5
Formaldehyde	ECPA	7	NA	L92	7	0	6
Formulation 7	Dow AgroSciences	2	BALB/c	L92	2	0	1
Hexyl cinnamic aldehyde	ECPA	5	NA	L92	5	0	5
Methyl 2-nonynoate	Ryan et al. (2000)	2	NA	80% EtOH	2	0	NA
Oxyflouren EC	ECPA	5	CBA	L92	3	2	2
Quinoxyfen/ cyproconazole	ECPA	6	CBA	L92	6	0	6
Trifluralin EC	ECPA	5	CBA	L92	5	0	6

Abbreviations: ACE = acetone; EC = emulsion concentrate; ECPA= European Crop Protection Association; EtOH = ethanol (diluent not specified); L92 = 1% aqueous Pluronic L92 1%; NA = not available; No. = number; SC = suspension concentrate

Table 5-11 Substances Tested in Multiple LLNA Studies in Aqueous Solutions with Discordant Results

Substance	Vehicle	Conc. (%)	SIs	Strain	EC3	Lab
1,4 Dihydroquinone	ACE/saline (1:1)	0.05, 0.1, 0.25, 0.5, 1.0	0.7, 1.0, 0.9, 1.9, 1.9	NA	NC	1
	ACE/saline (1:1)	0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10	1.4, 0.8, 1.2, 1.3, 1.9, 6.8, 10.9	NA	1.3	2
Oxyfluorfen EC	L92	1, 7, 33	0.81, 1.4, 4.9	CBA/Ca	30.8	1
	L92	1, 7, 33	0.9, 1.4, 2.8	CBA/J	NC	2
	L92	1, 7, 33	0.3, 0.9, 2.3	CBA/J	NC	3
	L92	1, 7, 33	1.1, 1.5, 3.1	CBA/JHsd	30.8	4
	L92	1, 7, 33	1.2, 1.2, 5.4	CBA/CaOlaHsd	18.1	5

Abbreviations: ACE = acetone; Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of 3; L92 = 1% aqueous Pluronic L92; LLNA = local lymph node assay; NA = Not available; NC = Not calculated since SI<3.0; SIs = stimulation indices

GP data were available for 24 substances (4 sensitizers/20 nonsensitizers in the GP) tested in aqueous solutions. These substances represented a total of 43 LLNA studies. Based on these comparative data, the LLNA has an accuracy of 54% (13/24), a sensitivity of 75% (3/4), a specificity of 50% (10/20), a false positive rate of 50% (10/20), and a false negative rate of 25% (1/4) (**Table 5-12**).

Table 5-12 Evaluation of the Performance of the LLNA for 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]
Pesticide Formulations and Pure Compounds Tested in Aqueous Solutions											
LLNA (CBA & BALB/c) vs. GP[3]	24	54	13/24	75	3/4	50	10/20	50	10/20	25	1/4
LLNA (CBA only) vs. GP[3]	21	57	12/21	75	3/4	53	9/17	47	8/17	25	1/4
LLNA (CBA only) 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	100	2/2	100	1/1	100	1/1	0	0/1	0	0/1
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. GP and human, and GP versus human is included here.

Eleven substances were discordant between the LLNA and the GP tests (**Table 5-13**). Ten of the 11 discordant substances (all overpredicted by the LLNA) were pesticide formulations tested in aqueous 1% Pluronic L92. These were the same 10 formulations noted in **Section 5.1.1.1**, where a detailed discussion of the discordant results is also detailed. The other discordant substance was neomycin sulfate, which was tested in 25% EtOH. Among the 11 of 24 substances classified as sensitizers by the LLNA that were classified as nonsensitizers in the GP (**Table 5-13**), 9/11 were based on BT results and 2/11 were based on GPMT results.

The one false negative substance based on LLNA results as compared to GP results, neomycin sulfate, was tested in the LLNA at a maximum concentration 12.5-fold lower than the induction concentration used in the guinea pig (**Table 5-13**). However, it should also be

noted that neomycin sulfate also gave a negative result in the LLNA when tested at 25% in DMSO, a non-aqueous vehicle (Basketter et al. 1994).

Table 5-13 Substances Tested in Aqueous Solution: Discordant Results Between the LLNA and GP

Substance Name	LLNA Results					GP Results			Skin Irritant?
	Vehicle	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Ind. Conc. (%)	Sens. Incid. (%)	Result[3]	
Atrazine SC	L92	100	7.3	36.4[4]	+	30	0	-[5]	Nonirritant at ≤ 25%[6]
BASF SE-1	L92	70	22.7	5.5	+	100	0	-[7]	Nonirritant at ≤ 50%[6]
EXP 11120 A	L92	100	5.3	64.9	+	100	0	-[7]	Nonirritant at 100%[6]
F & Fo WG 50 + 25	L92	25	15.2	0.003	+	30	0	-[7]	Nonirritant at ≤ 10%[6]
FAR01060-00	L92	100	3.6	88.5	+	100	0	-[7]	Nonirritant at 100%[6]
Formulation 2[8]	L92	80	15.8	15.7	+	NA	NA	-[7]	Nonirritant at 80%[9]
Formulation 7[8]	L92	100	3.2	85	+	100	0	-[7]	Nonirritant at 80%[9]
Fx + Me EW 69	L92	50	8.6	25.2	+	100	0	-[7]	Nonirritant at 100%[6]
Neomycin sulfate	25% EtOH	2	0.9	NC	-	25	76	+	Nonirritant at ≤ 25%[6]
Oxyfluorfen EC	L92	33	5.4	30.8[7]	+	10	26	-[5]	Nonirritant at ≤ 25%[6]
Trifluralin EC	L92	100	75.2	10.3[8]	+	50	10	-[7]	Nonirritant at ≤ 25%[6]

Abbreviations: Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of three; EW = emulsion, oil in water; GP = guinea pig test; Ind. Con. = induction concentration; L92 = 1% aqueous Pluronic L92; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI<3.0; SC = suspension concentrate; Sens. Incid. = sensitization incidence; SI = stimulation index; WG = water-dispersible granules

[1] Maximum concentration tested in the LLNA
[2] Maximum SI obtained in the LLNA
[3] (-) = nonsensitizer, (+) = sensitizer
[4] Mean value from 2 studies
[5] Guinea pig maximization test (GPMT) result
[6] Based on challenge concentration from a GPMT or Buehler test (BT)
[7] BT result
[8] LLNA conducted in BALB/c mice
[9] Based on irritation prescreen in mice
[10] Mean from 3 positive studies
[11] Mean of 5 studies

Among the substances tested in aqueous solutions, human data were available for only four (3 sensitizers/1 nonsensitizer in humans). Of these four, two were correctly identified by the LLNA when compared to human data. The accuracy statistics for the LLNA for this limited database are presented in **Table 5-12**.

Two substances, which had comparative human and GP data, were tested in aqueous solutions. Of these, one (neomycin sulfate) was correctly identified in the GP as a sensitizer,

compared to human results (Magnusson and Kligman 1969) (**Table 5-14**). Neomycin sulfate, when tested in aqueous solution (25% EtOH) in the LLNA (Gerberick et al. 1992) is false negative in the LLNA when compared to human results. As noted above, the maximum concentration of neomycin sulfate tested in the LLNA in aqueous solution (2%), is 12.5-fold less than the induction concentration (25%) used in both the GPMT and the HMT tests that gave positive results (Kligman 1966), but again, neomycin sulfate was also negative in the LLNA when tested at 25% in DMSO, a non-aqueous vehicle (Basketter et al.1994). The other substance for which there was both GP and human data, propylene glycol, was false negative in both the LLNA and the GPMT. It was classified as a sensitizer for this study based on its inclusion in a human patch test allergen test kit (ICCVAM 1999), along with the fact that Guillot et al. (1983) note anecdotal evidence of sensitization reactions in humans. However, there is published HMT data for propylene glycol that indicates it is a nonsensitizer (Kligman 1966; Guillot et al. 1983) and a weak human irritant (Basketter et al. 1997). The maximum concentration of propylene glycol that has been tested in humans is 25% (Kligman 1966). Given these uncertainties, this false negative result could be considered equivocal.

Table 5-14 Substances with Human Data Tested in Aqueous Solution

Substance Name	LLNA Results					GP Results				Human Results				Skin Irritant?
	Veh.	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Test	Ind. Conc (%)	Sens Incid (%)	Result[3]	Test	Ind. Conc (%)	Sens Incid (%)	Result[3]	
Butanol	H_2O	20	1.6[4]	NC	-	NA	NA	NA	NA	NA	NA	NA	-	NA
Methyl 2-nonynoate	80% EtOH	20	24.4	2.5	+	NA	NA	NA	NA	HRIPT	0.2	0	+	NA
Neomycin sulfate	25% EtOH	2	0.9	NC	-	GPMT	25	76	+	HMT	25	28	+	NA
Propylene glycol	H_2O	100	1.6	NC	-	GPMT[5]	1	0	-	--	--	--	+[6]	Nonirritant at 25%[7]

Abbreviations: Conc. = concentration; EC3 = estimated concentration needed to produce a stimulation index of three; EtOH = ethanol; GP = guinea pig; GPMT = guinea pig maximization test; HMT = human maximization test; HRIPT = human repeat insult patch test; Ind. = incidence; Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI<3.0; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle.

[1] Maximum concentration tested in the LLNA.

[2] Maximum SI obtained in the LLNA.

[3] (-) = nonsensitizer, (+) = sensitizer

[4] Test concentration that produced this SI was 5%.

[5] Also tested in Buehler test; Inc. Conc. = 0.2, Sens. Ind. = 0%

[6] Positive call on the basis that propylene glycol is included as a human patch test allergen (ICCVAM 1999).

[7] Test in humans.

5.3.2 *Medical Device Eluates Tested in Aqueous Solutions*

Of the 48 medical device eluates considered in this analysis, 100% (48/48) are LLNA negative. The constituents of these eluates were not provided by the submitter, so physico-chemical properties of any substances they contained are unknown. The submitted data are provided in **Appendix D3**.

None of these eluates had associated GP data or human data. All of the LLNA studies were reportedly done according to the ICCVAM-recommended protocol (ICCVAM 1999). The LLNA data provided by the submitter were average dpm for each treatment group (n = 5 animals); the individual animal data were not submitted (although the study report indicates that individual animal data were collected). SI values were calculated by NICEATM based on the submitted average values (**Appendix D3**).

The sample preparation for these samples was different that that for the pesticide formulations and pure substances discussed in **Section 5.3.1**. The test substances for the LLNA were eluates of medical devices prepared according to standard procedures (ASTM 2008, ISO 2002), rather than dilutions of specific substances. A concurrent positive control was included in each LLNA study. Another treatment group treated with an eluate sample spiked with a known sensitizer, 2,4-dinitrobenzenesulfonic acid, was also included in each LLNA study. The purpose of the spiked samples was reportedly to demonstrate that there was nothing present in the eluate that would attenuate a positive LLNA response.

These eluates were not analyzed to determine their constituents, or whether in fact any compound(s) were eluted from the medical device tested. Since the LLNA results were uniformly negative and no sample preparation control was included in the studies, the effectiveness of the sample preparation could not be determined, so the results from these eluates were not included with those from the pesticide formulations and pure substances discussed in **Section 5.3.1**.

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6.0 LLNA Data Quality

This section has been revised to include data received subsequent to the release of the draft addendum in January 2008. These data are summarized in **Section 2.0**.

Based on the available information, the published papers, and data submissions, information on compliance with GLP guidelines was available for data obtained from Dow AgroSciences, Dupont, 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 all of 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.

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7.0 Other Scientific Reports and Reviews

Six additional papers, identified since the publication of this addendum in January, 2008, have been added to this section.

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 pesticide formulations and other products, 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. (1999a)

Basketter et al. (1999a) 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; AOO]; DMSO: 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 Ikarashi et al. (2002)

The authors examined the sensitization potential of gold sodium thiosulfate (GST) in the GP and the mouse. GST has been included in a standard human patch test series, and the incidence of patients showing positive reactions to gold is increasing (contact allergy rates to gold were reported to be in the range 1–23% from various countries). GST was tested in the GPMT and in several *in vivo* assays in the mouse, including the mouse ear swelling test (MEST) (Gad et al. 1986), an ex-vivo variant of the LLNA, the sensitive LLNA (Ikarashi et al. 1993) and the mouse IgE test (Hilton et al. 1995, Dearman et al. 1992). GST was identified as a sensitizer in the GPMT (GST intradermal induction concentration, 1%; sensitization index 60% [6/10]. However, only 2/6 mice showed a positive response (ear swelling ≥ 20%) in the MEST, and GST did not induce an SI ≥ 3 in either variant of the LLNA. There was a significant difference in total serum IgE concentrations between vehicle- and GST-treated groups ($p < 0:05$). The authors concluded that GST was a weak sensitizer.

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, systemic allergic reactions and contact stomatitis. 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 GP 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 Tinkle et al. (2004)

The authors investigated the skin sensitization potential of beryllium, the cause of chronic beryllium disease, an incurable occupational lung disease that begins as a cell-mediated immune response to beryllium. Since occupational respiratory beryllium exposures have been decreasing and the rate of beryllium sensitization has not declined, the authors hypothesized that skin exposure to beryllium particles might be alternative route for sensitization. Optical scanning laser confocal microscopy and size-selected fluorospheres were used to demonstrate that ultrafine beryllium particles penetrate the stratum corneum of human skin, reaching the epidermis and, occasionally, the dermis. Skin sensitization in mice was suggested by peripheral blood and LN beryllium lymphocyte proliferation tests

(BeLPT), and by changes in LN T-cell activation markers, increased expression of CD44, and decreased CD62L following topical application of beryllium. Topically-applied beryllium also increased ear thickness in mice following challenge. The authors believe that these observations are consistent with development of a cell-mediated immune response following topical application of beryllium, and hypothesize a link between the persistent rate of occupational beryllium sensitization and skin exposure to ultrafine particles.

7.7 Shelnutt et al. (2007)

This is a review of the literature on the skin sensitization potential of hexavalent chromium. Hexavalent chromium is both a dermal irritant and a dermal sensitizer, causing ulceration of the skin and ACD. While the trivalent form of chromium is the naturally occurring valence, hexavalent chromium is one of the more prevalent sensitizers in the environment, present in detergents, cement, cosmetics, and foods. Research indicates that the hexavalent form exhibits greater skin-penetration properties than the trivalent form, although it is hypothesized that hexavalent chromium is transformed to trivalent chromium in the body and it is the trivalent form that induces sensitization. Repeated exposure to 4–25 ppm of hexavalent chromium can both cause sensitization and elicit ACD. Exposure to 20 ppm hexavalent chromium can cause skin ulcers in nonsensitized people. Chromium ACD can be persistent and debilitating, perhaps because of the high prevalence and ubiquity of hexavalent chromium.

7.8 Chipinda et al. (2008)

Zinc diethyldithiocarbamate (ZDEC) and its disulfide, tetraethylthiuram disulfide (TETD) occur in rubber products, and are well-documented contact sensitizers in animals and humans. They are cross-reactive, as sensitization to one often confers sensitization to the other. This paper explored haptenation mechanisms of ZDEC by using high performance liquid chromatography and mass spectrometry to identify ZDEC oxidation/reduction products and sites of protein binding. The LLNA was employed to test ZDEC and its oxidation products for sensitization potential and to and examine possible mechanisms of hapten formation via elimination of oxidation and chelation mechanisms by substituting cobalt for zinc in ZDEC, to produce CoDEC. Oxidation of ZDEC produced TETD, tetraethylthiocarbamoyl disulfide, and tetraethyldicarbamoyl disulfide (TEDCD). The LLNA

identified ZDEC, sodium diethyldithiocarbamate, TEDCD, and TETD as sensitizers, and CoDEC, as a nonsensitizer. While ZDEC bound to the copper-containing active site of superoxide dismutase, CoDec did not, suggesting chelation of metal containing proteins as a possible mechanism of hapten formation.

7.9 Fukuyama et al. (2008)

The authors used the LLNA to test the sensitization potential of chromated copper arsenate (CCA), a commonly used wood preservative, and its components, for sensitization potential. LLNA studies were done using both AOO and DMSO as vehicles. CCA components tested included As_2O_5, CrO_3, and CuO_2. Trimellitic anhydride in AOO was used as a positive control. All metal compounds were detected as sensitizers by the LLNA. EC3 values for metal compounds tested in AOO and DMSO were different (CCA: EC3 in AOO = 1.86%, EC3 in DMSO < 0.3%; As_2O_5: EC3 in AOO = 0.8%, EC3 in DMSO < 0.3%). CuO_2 (EC3 = 1.69%) and CrO_3 (EC3 < 0.3%) were tested in DMSO only. ATP was also measured in an aliquot of the lymph node suspension via a luciferin-luciferase assay, and found to increase with increasing dose of the metal compounds.

7.10 Jowsey et al. (2008)

The authors conducted a retrospective examination of LLNA data in AOO for 18 substances that had been tested multiple times in AOO (2 - 15 studies per substance) to determine the inherent variability in the calculated EC3 values. The highest observed variability was for isoeugenol (31 studies) at 4.1-fold. A second retrospective analysis of data from the literature and previously unpublished studies for 18 substances that had been tested in the LLNA using at least two of 15 different vehicles was conducted. For 6/18 substances (ethylene glycol dimethacrylate, eugenol, geraniol, imidazolidinyl urea, hydroxycitronellal, and nickel sulfate), the variability was less than 5-fold. For 6/18 chemicals (3-dimethylaminopropylamine, cinnamic aldehyde, isoeugenol, p-tert-butyl-a-ethyl hydrocinnamal, methylchloroisothiazolinone/methylisothiazolinone, and potassium dichromate), the variability was greater than 5-fold but less than 10-fold. For 6/18 chemicals (dinitrobenzene sulfonate, 1,4-hydroquinone, 1,4-phenylenediamine, methyldibromoglutaronitrile, formaldehyde, and glutaraldehyde), the observed range was greater than 10-fold. Further examination of the data for the substances in the highest-

variability group suggested that the high variability might be due to an underestimation of potency in the LLNA associated with the use of predominantly aqueous vehicles or propylene glycol. In contrast, use of AOO, DMF, methyl ethyl ketone, DMSO, and 9:1 ethanol:water resulted in less variable potency estimates for most substances.

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