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CONSUMER PRODUCT SAFETY COMMISSION
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BALLOT VOTE SHEET

Date: January 19, 2011

TO : The Commission
Todd A. Stevenson, Secretary

THROUGH: Cheryl A. Falvey, General Counsel
Kenneth R. Hinson, Executive Director

SUBJECT : Staff Response to the ICCVAM Recommendations on Revisions to the Murine Local Lymph Node Assay

BALLOT VOTE due: January 26, 2011

The attached memorandum from the Health Sciences Directorate summarizes the recommendations of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) regarding the Murine Local Lymph Node Assay, a method for determining sensitizing potential. The staff recommends that the Commission accept the ICCVAM recommendations and instruct the staff to so inform ICCVAM by letter.

Please indicate your vote.

I. Accept the ICCVAM recommendations and instruct the staff to so inform ICCVAM by letter.

_____ _____
Signature Date

II. Reject the ICCVAM recommendations and instruct the staff to so inform ICCVAM by letter.

_____ _____
Signature Date

Attachment - *Staff Response to the ICCVAM Recommendations on Revisions to the Murine Local Lymph Node Assay, a Method for Determining Sensitizing Potential*, memorandum from Joanna Matheson, Ph.D., Directorate for Health Sciences, to the Commission, January 5, 2011.

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Memorandum

Date: January 5, 2011

TO : The Commission
Todd A. Stevenson, Secretary

THROUGH: Kenneth R. Hinson, Executive Director
Cheryl A. Falvey, General Counsel

FROM : Robert J. Howell, Assistant Executive Director
Office of Hazard Identification and Reduction
Joanna Matheson, Ph.D., Toxicologist
Directorate for Health Sciences

SUBJECT : Staff Response to the ICCVAM Recommendations on Revisions to the Murine Local Lymph Node Assay, a Method for Determining Sensitizing Potential

This memorandum discusses the recommendations of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) regarding the Murine Local Lymph Node Assay (LLNA), including two nonradioactive versions of the assay: (1) the Bromodeoxyuridine Enzyme-linked Immunosorbent Assay (BrdU-ELISA), and (2) the Daicel Chemical Industries version (LLNA:DA), as well as (3) an update on the LLNA's applicability domain, particularly its effectiveness in testing pesticide formulations, metals, and substances in aqueous solutions. In addition, information is provided on whether these revisions are acceptable in the regulatory context for the purpose of classification for labeling under the Federal Hazardous Substances Act (FHSA) (15 U.S.C. 1261-1278).

I. Introduction

A. Background

The National Institutes of Health Revitalization Act of 1993 directed the National Institute of Environmental Health Science (NIEHS) to establish a method and criteria for the validation and regulatory acceptance of alternative testing methods (Public Law No. 103-43, Section 1301). To accomplish these goals, NIEHS created an ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), which was made permanent by the ICCVAM Authorization Act of 2000 (Public Law 106-545). The Committee is composed of representatives from 15 federal regulatory and research agencies; these agencies generate, use, or provide information from toxicity test methods for risk assessment purposes. The duties of ICCVAM are to review, optimize, and validate new, revised, or alternative test methods that encourage the reduction, refinement, or replacement of the use of animals in testing. In addition,

ICCVAM is to provide test recommendations to federal agencies and other stakeholders to facilitate appropriate interagency and international harmonization of toxicological test protocols. In 1998, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) was established to assist ICCVAM in performing the activities necessary for the validation and regulatory acceptance of alternative test methods. ICCVAM submits test recommendations for a test method to federal agencies that require or recommend acute or chronic toxicological testing. According to Public Law 106-545, these agencies should promote and encourage the development and use of alternatives to animal test methods for regulatory purposes, and ensure that any new or revised acute or chronic toxicity test method is valid for its proposed use under the mandate of the ICCVAM Authorization Act of 2000. Federal agencies have 180 days to identify any relevant test methods for which the ICCVAM test recommendations may be added or substituted, review such test recommendations, and notify ICCVAM if they will adopt the ICCVAM test recommendations.

On June 25, 2010, ICCVAM forwarded three recommendations to the Commission for action: (1) a nonradioactive form of the LLNA, the BrdU-ELISA; (2) a nonradioactive form of the LLNA, the LLNA:DA; and (3) an expanded applicability domain of the LLNA. The CPSC needs to determine if either of the proposed alternative versions of the LLNA and the expanded applicability domain would be acceptable under the Federal Hazardous Substances Act (FHSA). Under the mandate of the ICCVAM Authorization Act of 2000, federal agencies have 180 days to identify any relevant test methods for which the ICCVAM test recommendations may be added or substituted, review such test recommendations, and notify ICCVAM if they will adopt the ICCVAM test recommendations. ICCVAM had been informed by CPSC staff that the Commission's vote will not meet the December 15, 2010 deadline, but will be done as quickly as possible to reduce the length of the delay.

B. Validation of Alternative Methods

Validation of alternative methods is required before regulatory acceptance and utilization by federal agencies. In general, for an alternative method to be considered valid, it must be reliable (i.e., the toxicity predictions of test substances are repeatable within the same laboratory and reproducible across/among different laboratories) and relevant (i.e., the alternative test method is useful for measuring the biological effect of interest such as sensitization).

The reliability and relevancy of an alternative test method can be assessed from the statistical analysis of data. The relevance of an alternative test method can be determined by comparing the performance of the alternative test to the test that it is designed to replace. Performance is typically evaluated by calculating the accuracy,[1] false positive rate,[2] false negative rate,[3] sensitivity,[4] or specificity[5] of the alternative test method. The reliability of the alternative test method can be determined from the reproducibility of test method results within and among laboratories.

[1] Accuracy - proportion of correct outcomes.
[2] False positive rate - proportion of all negative substances that are falsely identified as positive.
[3] False negative rate - proportion of all positive substances that are falsely identified as negative.
[4] Sensitivity – the proportion of all positive substances that are classified as positive.
[5] Specificity – the proportion of all negative substances that are classified as negative.

C. Federal Hazardous Substances Act Requirements

Cautionary labeling of hazardous household substances is mandated by the FHSA. Under the FHSA, to be a hazardous substance, a product must present one or more of the hazards enumerated in the statute, and it must have the potential to cause substantial personal injury or substantial illness during, or as a result of, any customary or reasonably foreseeable handling or use.

FHSA "Strong Sensitizer": "Strong sensitizers" are one of the seven hazards defined under the FHSA. The definition of "*strong sensitizer,"* which appears in section 2(k) of the FHSA (15 U.S.C. §1262(k), and restated in 16 CFR 1500.3(b)(9)) is:

> *Strong sensitizer means a substance which will cause on normal living tissue through an allergic or photodynamic process a hypersensitivity which becomes evident on reapplication of the same substance and which is designated as such by the Commission. Before designating any substance as a strong sensitizer, the Commission, upon consideration of the frequency of occurrence and severity of the reaction, shall find that the substance has significant potential for causing hypersensitivity.*

Five substances have been identified in the FHSA as strong sensitizers[6]: (1) paraphenylenediamine and products containing it; (2) powdered orris root and products containing it; (3) epoxy resin systems containing in any concentration ethylenediamine, diethylenetriamine, and diglycidyl ethers of molecular weight less than 200; (4) formaldehyde and products containing 1 percent or more of formaldehyde; and (5) oil of bergamot and products containing 2 percent or more of oil of bergamot. These designated compounds were transferred over when the CPSC was established.

Since its inception in 1972, the CPSC has not designated any substances to be strong sensitizers. However, in 1986, the Commission issued a rule clarifying the FHSA's "strong sensitizer" definition with supplemental definitions as recommended by a Technical Advisory Panel on Allergic Sensitization (TAPAS).[7] The following supplemental definitions were intended to clarify the interpretation of the statutory definition of a "strong sensitizer":

> - *Sensitizer: A sensitizer is a substance that will induce an immunologically-mediated (allergic) response, including allergic photosensitivity. This allergic reaction will become evident upon re-exposure to the same substance. Occasionally, a sensitizer will induce and elicit an allergic response on first exposure by virtue of active sensitization.*
>
> - *Strong: In determining that a substance is a "strong" sensitizer, the Commission shall consider the available data for a number of factors. These factors should include any or all of the following (if available):*
>   - *Quantitative or qualitative risk assessment*

[6] 16 CFR §1500.13.

[7] 16 CFR §1500.3(c)(5).

- *Frequency of occurrence and range of severity of reactions in healthy or susceptible populations*
- *The result of experimental assays in animals or humans (considering dose-response factors), with human data taking precedence over animal data*
- *Other data on potency or bioavailability of sensitizers*
- *Data on reactions to a cross-reacting substance or to a chemical that metabolizes or degrades to form the same or a cross-reacting substance*
- *The threshold of human sensitivity*
- *Epidemiological studies*
- *Case histories*
- *Occupational studies*
- *Other appropriate in vivo and in vitro test studies*

- Severity of Reaction: The minimal severity of a reaction for the purpose of designating a material as a "strong sensitizer" is a clinically important reaction. For example, strong sensitizers may produce substantial illness, including any or all of the following:

- *Physical discomfort*
- *Distress*
- *Hardship*
- *Functional or structural impairment*

These may, but not necessarily, require medical treatment or produce loss of functional activities.

- Significant potential for causing hypersensitivity: "Significant potential for causing hypersensitivity" is a relative determination that must be made separately for each substance. It may be based on chemical or functional properties of the substance, documented medical evidence of allergic reactions obtained from epidemiological surveys or individual case reports, controlled in vitro or in vivo experimental assays, or susceptibility profiles in normal or allergic subjects.

- Normal living tissue: The allergic hypersensitivity reaction occurs in normal living tissues, including the skin and other organ systems, such as the respiratory or gastrointestinal tract, either singularly or in combination, following sensitization by contact, ingestion or inhalation.

While the FHSA does not require manufacturers to perform any specific battery of toxicological tests to assess the potential risk of chronic hazards, the manufacturer is required to label a product appropriately, according to the FHSA requirements; with the exception that if the product is a toy or other article intended for use by children and is a hazardous substance, then the product is, by definition, a banned hazardous substance, unless specifically exempted.[8] When determining if a consumer product, which is composed of a mixture of substances, is a hazardous substance, the mixture should be tested—and not the individual components of the mixture—because synergistic or antagonistic reactions may lead to erroneous determinations concerning the toxic, irritant, and corrosive properties of the substance (16 CFR § 1500.5).

[8] 16 C.F.R. §1500.3(b)(15)(i).

Sensitizers in Art Materials: Congress amended the FHSA in 1988, to include the Labeling of Hazardous Art Materials Act (LHAMA) requirements. The LHAMA requires a reviewing procedure for developing precautionary labels for all art materials. This amendment to the FHSA concerns chronic health hazards known to be associated with a product or product component when present in a physical form, volume, or concentration that presents the potential to produce a chronic health hazard as determined by a toxicologist. Within the regulation under the Act, a "sensitizer" is defined as *a substance known to cause, through an allergic process, a chronic adverse health effect which becomes evident in a significant number of people on re-exposure to the same substance.*[9] To protect users from known sensitizers found within art materials, each label shall contain a list of those sensitizers present in sufficient amounts to contribute significantly to a known skin or respiratory sensitization.[10]

D. Past and Current Sensitization Testing

Data on the sensitization potential of some chemicals comes from studies using human volunteers, and the development of animal sensitization tests has been based on a comparison to the human tests performed with the same chemicals. Two approaches for predictive sensitization testing in humans that have been in use are the Human Maximization Test (HMT) and the Human Repeated Insult Patch Tests (HRIPT). These tests vary with regard to the number of induction patch tests, the placing of the patches, and the use of a maximization step. The HMT is no longer in use due to ethical concerns about its potential health consequences. Contract laboratories have performed the vast majority of human sensitization tests and the scientific literature contains a limited number of publications giving results from tests with cosmetic ingredients such as preservatives and fragrance chemicals.

Historically, the Guinea Pig Maximization Test (GPMT) and the Buehler Assay (BA) have been the primary animal assays used to determine the sensitizing ability of a chemical. The GPMT is a highly sensitive method; however, some of the sensitivity arises due to the co-administration of a painful immune stimulant. It includes both intra-dermal and topical induction treatments. The BA uses repeat closed topical applications. The GPMT is regarded as a more sensitive assay that may also, for certain substances, overestimate the sensitization hazard for the compound tested. The Buehler test is less sensitive and may underestimate the sensitization potential of a compound.

In 1997, the LLNA was proposed to ICCVAM as a stand-alone alternative method to the GPMT and the BA for hazard identification. In 1999, based on the validation database and performance, ICCVAM recommended the LLNA as an alternative test method for assessing the skin sensitization potential of most types of substances. The consensus of the peer review panel was that the LLNA performed as well as the GPMT and BA for hazard identification of strong to moderate chemical sensitizing [dermal] agents but lacked strength in accurately predicting some weak sensitizers and some strong irritants. The LLNA provides several advantages compared to the guinea pig assays, including elimination of potential pain and distress, use of fewer animals, shorter test duration, a more objective end point, less test substance required, and the availability of dose-response information. United States regulatory agencies accepted the LLNA as a valid alternative test method for allergic contact dermatitis testing. The LLNA was adopted as a test

[9] 16 C.F.R. §1500.14(b)(8)(i)(B)(9).
[10] 16 C.F.R. §1500.14(b)(8)(i)(E)(5).

guideline (test guideline [TG] 429) in 2002, by the Organization for Economic and Cooperative Development (OECD) after the ICCVAM validation of the assay.

In the intervening years, the National Toxicology Program (NTP) has used extensively the assay to study chemical hypersensitivity based upon its acceptance as a stand-alone alternative. The U.S. Environmental Protection Agency (EPA) indicates that the LLNA, along with the GPMT and BA, are acceptable test methods, with the LLNA as a preferred alternative method, where applicable, to the guinea pig tests. The U.S. Food and Drug Administration (FDA), in its Guidance for Industry, indicates that the sensitizing potential of a drug should be screened using an appropriate test, such as the GPMT, BA, LLNA, the guinea pig inhalation induction and challenge assay, or other appropriate alternative assays.[11]

II. Alternative Tests for Sensitization, ICCVAM Recommendations

Currently, no *in vitro* or *in silico*[12] systems have undergone validation for determining sensitizing potential. Both approaches are evolving methodologies and are being pursued to reduce the number of expensive laboratory and animal experiments performed.

The remainder of Section II of this memo will describe each of the submitted ICCVAM recommendations, relevant validation and performance data, and ICCVAM conclusions.

A. LLNA BrdU-ELISA

1. Background

The LLNA is a test method developed to assess the potential of a test substance to induce allergic contact dermatitis in humans. The basic principle underlying the LLNA is that sensitizers induce a proliferation of lymphocytes in the lymph node draining the site of substance application. Under appropriate test conditions, this proliferation is proportional to the dose applied and provides a means of obtaining an objective measurement of sensitization. The LLNA was the first test method evaluated and recommended by ICCVAM. As stated earlier, the advantages of this test method include that it uses fewer animals, provides dose-response information, and eliminates pain and distress compared to the guinea pig assays. In 2001, following a comprehensive independent peer review of the LLNA, ICCVAM developed recommendations applicable to the regulatory use of the LLNA and prepared a recommended protocol. In March 2008, an international peer review panel (Panel) composed of expert scientists from industry, academia, and other scientific professionals organized by ICCVAM, in collaboration with NICEATM, convened to review and evaluate the validation status, make recommendations for revisions, and provide final comments on the usefulness and limitations of proposed modifications to the LLNA. The Panel provided conclusions and recommendations in its reports. ICCVAM subsequently considered the Panel's conclusions and recommendations, as well as comments from the Scientific Advisory Committee on

[11] Guidance for Industry. Immuntoxicology Evaluation of Investigational New Drugs, October 2002, Center for Drug Evaluation and Research.

[12] *In silico* data is a computational approach, using sophisticated computer models for the determination of a sensitizing potential.

Alternative Toxicological Methods (SACATM) and the public, and updated the 2001 ICCVAM-recommended LLNA protocol. On March 9, 2010, the Commission voted unanimously to approve three ICCVAM recommendations regarding the LLNA including: (1) updates to the test method protocol; (2) establishment of performance standards; and (3) a modified form of the assay, the reduced Local Lymph Node Assay (rLLNA).

The BrdU-ELISA was developed by Dr. Masahiro Takayoshi at the Chemicals Evaluation and Research Institute in Saitama, Japan. Since the BrdU-ELISA does not require a radioactive marker, it can be used by laboratories that cannot use the traditional LLNA because they either do not have a license for using radioisotopes or their countries, such as Japan, discourage or severely limit the use of radioactive materials. The BrdU-ELISA method is mechanistically and functionally identical to the traditional LLNA. The sole difference is that the BrdU-ELISA assesses cell proliferation using the incorporation of BrdU into newly synthesized DNA, rather than by quantifying the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine, as is done in the traditional LLNA. The increase in BrdU in the lymph nodes then is quantified colorimetrically using a commercially available ELISA kit. For the March 2008 Panel meeting, ICCVAM examined data for 24 substances tested in a single laboratory. The Panel agreed with the ICCVAM draft recommendations that the BrdU-ELISA may be useful for identifying potential sensitizing substances, but noted that a detailed test method protocol, individual animal data on a larger set of balanced reference substances, and an evaluation of inter-laboratory reproducibility were needed before definitive conclusions could be made. Subsequent to the March 2008 Panel meeting, NICEATM obtained a detailed test method protocol and additional data, as well as an evaluation of inter-laboratory reproducibility. The Panel reconvened in public session in April 2009, to review the ICCVAM-revised background review documents and to finalize its conclusions and recommendations on the BrdU-ELISA.

2. Validation and Performance

Modified method protocols are expected to achieve a level of performance that is equivalent to or exceeds the accuracy and reliability of the traditional LLNA for identifying sensitizers. Test method reliability is the degree to which a test method can be performed reproducibly within and among laboratories over time (intra-laboratory repeatability, and intra-laboratory and inter-laboratory reproducibility). Subsequent to the April 2009 meeting, NICEATM received results on 12 additional substances, bringing the total substances evaluated to 43. The evaluation compared the performance of the BrdU-ELISA with results from the validated traditional LLNA, guinea pig, and human data (if available).

Accuracy: for this performance analysis, results for 12 additional substances were received by NICEATM after the April 2009 Panel evaluation. The validation database was comprised of 43 substances. The overall accuracy of the BrdU-ELISA was 95 percent. The BrdU-ELISA correctly identified all 32 LLNA sensitizers (thus, 0 percent false negatives) and 9 of 11 LLNA nonsensitizers (thus, an 18 percent false positive rate).

Reliability: the extent of agreement among laboratories (inter-laboratory reproducibility) in assigning the same sensitization classification by the BrdU-ELISA was assessed in three to seven laboratories for 10 substances. There was 100 percent inter-laboratory agreement for nine substances (7 sensitizers and 2 non-sensitizers). One of the nonsensitizers produced false positive results. The reproducibility of classification of 18 substances was evaluated in 2 to 18 repeat tests. The results for 85 percent of the sensitizers were 100 percent concordant and 60 percent of the nonsensitizers were 100 percent concordant. Overall, 78 percent of the 18 substances were 100 percent concordant. Intra-laboratory reproducibility demonstrated 100 percent concordance for 10 of 12 substances. The two remaining substances, nonsensitizers, were false positives.

3. Recommendations for the BrdU-ELISA

The Panel reconvened in April 2009, to review and evaluate the revised background review document and ICCVAM recommendations. The Panel provided final comments on the usefulness and limitations of the BrdU-ELISA in their June 2009 report. The Panel supported the revised draft ICCVAM recommendations that the BrdU-ELISA can be used for identifying substances as potential skin sensitizers and nonsensitizers. The Panel considered the database of substances analyzed to be representative of a sufficient range of chemicals and concluded that the accuracy analysis had made appropriate comparisons to the traditional LLNA, guinea pig tests, and human data/experience. The Panel stated that:

- *A stimulation index (SI) ≥ 2.0[13] should be used to identify substances as sensitizers and SI of < 1.3 should be used to identify non-sensitizers. A limitation of the BrdU-ELISA involves the indeterminate identification of substances that produce an SI greater than or equal to 1.3 but less than 2.0.*
- *There should be analyses of all non-radioactive LLNA methods of the process and results used to define the cutoff values (the SI values) and a protocol should be developed for evaluating such cutoffs that future developers could apply during development of new methods.*
- *Additional studies should be conducted for other substances, including metals, irritants, and formulations, that have comparative human, guinea pig, and traditional LLNA data. While specifically applicable to the BrdU-ELISA, these data needs are common to all variants of the LLNA. Regarding irritants, the proposed future studies might help explain why results obtained using the BrdU-ELISA and traditional LLNA were discordant, and further address the general challenge of discriminating irritants in the traditional LLNA itself.*

Subsequent to the Panel meeting, ICCVAM's Immunotoxicity Working Group (IWG), along with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) liaisons and the OECD Expert Group, considered the Panel's conclusions and recommendations, as well as those from the public and the SACATM. A series of meetings were convened throughout the summer and fall of 2009, concluding in an international harmonization

[13]A stimulation index of 3.0, a threefold increase in proliferation, is used to discriminate between sensitizers and nonsensitizers in the traditional LLNA assay.

meeting in October 2009, with the goal harmonizing the decision criteria with TG429 (the traditional LLNA) with respect to how a positive result is identified. A new single SI value was proposed, focusing on avoiding false negative results, while leaving only a narrow range where weakly positive results may be false positives.

ICCVAM recommended the following future studies to further characterize the usefulness and limitations of the BrdU-ELISA:

- *Efforts should be made to identify additional human data, human experience, and data on nonsensitizing skin irritants to further assess the usefulness and limitations of this method for identifying human sensitizing substances and for the impact of these substances on the false positive rate of the BrdU-ELISA.*
- *Efforts should be made to further characterize the sensitization potential of borderline positive substances (those substances with SI values falling between 1.6 and 1.9), to determine whether such results are false positives. These efforts could include non-animal data, such as evaluations of peptide reactivity; determination of molecular weight; results from related chemicals; human studies, where ethically and scientifically justified; review of occupational exposures; and postmarketing experience, or in vitro testing. All decision criteria should be reassessed as additional discriminators and data become available.*

4. ICCVAM Conclusion

ICCVAM concluded that the scientific validity of the BrdU-ELISA has been evaluated adequately and supports the use of the BrdU-ELISA to distinguish between skin sensitizers and nonsensitizers. ICCVAM recommends that a SI $\geq$ 1.6 be used as the decision criteria to identify substances as potential sensitizers; this recommendation is based on the fact that no false negatives occurred when this SI value was used.

There is a small possibility of false positive results when borderline positive responses between SI values of 1.6 and 1.9 are obtained. For a weakly positive response, which falls within this range, users may want to consider additional information, such as the strength of the dose-response relationship; evidence of systemic toxicity, or excessive irritation; and where appropriate, statistical significance together with the SI values, to confirm that such results are positive.

ICCVAM considers the applicability domain for the BrdU-ELISA to be the same as the traditional LLNA, unless there are properties associated with a class of materials that may interfere with the accuracy of the BrdU-ELISA. One exception would be nickel compounds where, unlike the traditional LLNA, the BrdU-ELISA correctly was able to identify them as potential sensitizers.

Following the Panel peer review meetings in 2008 and 2009, ICCVAM finalized its conclusions and recommendations for the BrdU-ELISA uses and limitations; the test protocol; the test method performance standards; and future studies. Based upon these activities, a draft test guideline for the BrdU-ELISA was submitted in July 2009, to the

OECD member countries for review. CPSC staff and ICCVAM cohosted an OECD Expert Consultation meeting in October 2009, and a conference call in January 2010, to evaluate member country comments on the draft test guideline. A final draft test guideline was forwarded to the OECD to consider for adoption at their March 2010 meeting. The new test guideline, designated Test Guideline 442B, was adopted by the OECD in July 2010.

5. CPSC Staff Recommendation

Staff agrees with the ICCVAM recommendations on the BrdU-ELISA, with its utility in determining the sensitizing capacity of a substance. This modified form of the traditional LLNA also addresses animal welfare considerations by providing nonradioactive methods that can reduce the number of animals needed per test, as well as result in less pain and suffering when used in place of the traditional guinea pig test methods for countries that severely limit or discourage the use of radioactive materials, materials which are required by the traditional LLNA. Furthermore, under the current database, the stimulation index recommended for this test method avoids false negatives.

B. LLNA:DA

1. Background

The LLNA:DA was developed by Dr. Kenji Idehara at Daicel Chemical Industries in Hyogo, Japan. Similar to the BrdU-ELISA, the LLNA:DA does not require a radioactive marker; therefore, it can be used by laboratories that cannot use the traditional LLNA because they either do not have a license for using radioisotopes or their countries, such as Japan, discourage or severely limit the use of radioactive materials. The mechanistic basis of the LLNA:DA is identical to that of the traditional LLNA. However, the LLNA:DA differs from the traditional LLNA in test substance treatment and sampling schedule. The LLNA:DA assesses cell proliferation by detecting increases in adenosine triphosphate (ATP) as an indicator of cell number at the end of cell proliferation rather than by quantifying the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine as is done in the traditional LLNA. The increase in ATP content in the lymph nodes is then quantified using bioluminescence (a luciferin-luciferase assay). The emitted light intensity is linearly related to the ATP concentration. The luciferin-luciferase assay is a sensitive method for ATP quantitation that has been used in a wide variety of applications. For the March 2008 Panel meeting, ICCVAM examined data for 29 substances tested in a single laboratory. The Panel agreed with the ICCVAM draft recommendations that the LLNA:DA may be useful for identifying potential sensitizing substances but noted that a detailed test method protocol, individual animal data on a larger set of balanced reference substances, and an evaluation of inter-laboratory reproducibility were needed before definitive conclusions could be made. The Panel viewed the aforementioned treatment schedule difference between the LLNA:DA and the traditional LLNA to be potentially significant if the LLNA:DA induced the elicitation phase of skin sensitization. The Panel was also concerned that the 1 percent sodium lauryl sulfate (SLS) pretreatment step might modify the inherent sensitivity of the LLNA. Subsequent to the March 2008 Panel meeting, NICEATM obtained a detailed test method protocol and additional data, as well as an evaluation of inter-laboratory reproducibility.

The Panel reconvened in public session in April 2009, to review the ICCVAM-revised background review documents and to finalize its conclusions and recommendations on the LLNA:DA.

2. Validation and Performance

Modified method protocols are expected to achieve a level of performance that is equivalent to or exceeds the accuracy and reliability of the traditional LLNA for identifying sensitizers. Test method reliability is the degree to which a test method can be performed reproducibly within and among laboratories over time (intra-laboratory repeatability, intra-laboratory and inter-laboratory reproducibility). Subsequent to the April 2009 meeting, NICEATM received results on 15 additional substances, bringing the total substances evaluated to 44. The evaluation compared the performance of the LLNA:DA with results from the validated traditional LLNA, guinea pig tests, and human data/experience (if available).

Accuracy: for this performance analysis, the ability of the LLNA:DA to identify potential skin sensitizers was compared to that of the traditional LLNA. For the validation database of 44 substances, the LLNA:DA correctly identified all 32 LLNA sensitizers (thus 0 percent false negatives) and 9 of 12 LLNA nonsensitizers (thus a false positive rate of 25 percent). The LLNA:DA had an overall accuracy of 93 percent compared to the traditional LLNA.

Reliability: the extent of agreement among laboratories (inter-laboratory reproducibility) in assigning the same sensitization classification by the LLNA:DA was assessed in 3 to 10 laboratories for 12 substances. There was 100 percent inter-laboratory agreement for 10 substances (7 sensitizers and 3 non-sensitizers). There was 67 percent agreement for the remaining two traditional LLNA sensitizers. A second phase inter-laboratory validation study evaluated 5 substances in 4 to 7 laboratories. There was 100 percent inter-laboratory agreement for 4 substances (3 sensitizers and 1 nonsensitizer), and 75 percent agreement for the remaining traditional LLNA sensitizer. The reproducibility of classification of 14 substances was evaluated in 3 to 18 repeat tests. The results for 80 percent of the sensitizers were 100 percent concordant and 75 percent of the nonsensitizers were 100 percent concordant.

3. Recommendations for the LLNA:DA

The Panel reconvened in April 2009, to review and evaluate the revised background review document and ICCVAM recommendations. The Panel provided final comments on the usefulness and limitations of the LLNA:DA in their June 2009 report. The Panel supported the revised draft ICCVAM recommendations that the LLNA:DA can be used for identifying substances as potential skin sensitizers and nonsensitizers. The Panel considered the database of substances analyzed to be representative of a sufficient range of chemicals and concluded that the accuracy analysis had made appropriate comparisons to the traditional LLNA, guinea pig tests, and human data/experience. The Panel concluded the following with regard to the LLNA:DA:

- *A stimulation index (SI) ≥ 2.5 should be used to identify substances as sensitizers and SI of ≤ 1.7 should be used to identify non-sensitizers. A limitation of the LLNA:DA involves the indeterminate identification of substances that produce an SI greater than or equal to 1.7 but less than 2.5.*
- *There should be analyses of all non-radioactive LLNA methods of the process and results used to define the cutoff values (the SI values) and a protocol should be developed for evaluating such cutoffs that future developers could apply during development of new methods.*
- *Noted that supplemental power calculations for the LLNA:DA test method indicated that the power for detecting a 3-fold increase in the treatment group was estimated to be 95% for a sample size of 3 mice per dose group. Thus, the Panel identified the use of 3 animals per dose group as a potential opportunity to reduce animal number when using modified assays in the future.*
- *Additional studies should be conducted for other substances, including metals, irritants, and formulations, that have comparative human, guinea pig and traditional LLNA data. Regarding irritants, the proposed future studies might help explain why results obtained using the LLNA:DA and traditional LLNA were discordant, and further address the general challenge of discriminating irritants in the traditional LLNA itself.*
- *The Panel disagreed with the revised ICCVAM draft recommendation that a separate performance standard be developed to assess modified versions of the LLNA:DA test method. Although the test methods differ in the dosing regimen and in the timing of the assay, the Panel viewed the LLNA:DA as mechanistically similar to the traditional LLNA, in that both methods measure cellular stimulation in the draining lymph node. Consequently the ICCVAM-recommended performance standards are applicable to the LLNA:DA as a mechanistically and functionally similar test method.*

Subsequent to the Panel meeting, the ICCVAM's IWG, along with ECVAM and JaCVAM liaisons and the OECD Expert Group, considered the Panel's conclusions and recommendations, as well as those from the public and the SACATM. A series of meetings were convened throughout the summer and fall of 2009, concluding in an international harmonization meeting in October 2009, with the goal of harmonizing the decision criteria with TG429 (the traditional LLNA) with respect to how a positive result is identified. The OECD Expert Group agreed with ICCVAM that SI ≥ 1.8 provided optimal test method performance by preventing false negative results.

ICCVAM recommended the following future studies to further characterize the usefulness and limitations of the LLNA:DA:

- *Efforts should be made to identify additional human data, human experience, and data on nonsensitizing skin irritants to further assess the usefulness and limitations of this method for identifying human sensitizing substances and for the impact of these substances on the false positive rate of the LLNA:DA.*
- *Efforts should be made to further characterize the sensitization potential of borderline positive substances (those substances with SI values falling between 1.8 and 2.5), to determine whether such results are false positive. These efforts could include non-animal data, such as evaluations of peptide reactivity; determination of molecular weight; results from related chemicals; human studies, where ethically and*

scientifically justified; review of occupational exposures and post-marketing experience, or in vitro testing. All decision criteria should be reassessed as additional discriminators and data become available.

- *Inconsistent results for nickel sulfate suggest that the LLNA:DA may not be suitable for testing nickel compounds. Additional data on such compounds with comparative human or guinea pig data is needed to evaluate more comprehensively the suitability of the LLNA:DA for testing nickel compounds.*

4. ICCVAM Conclusion

ICCVAM concluded that the scientific validity of the LLNA:DA has been evaluated adequately and supports the use of the LLNA:DA to distinguish between skin sensitizers and nonsensitizers. ICCVAM recommends that a SI $\geq$ 1.8 be used as the decision criteria to identify substances as potential sensitizers; this recommendation is based on the fact that no false negatives occurred when this SI value was used.

There is a possibility of false positive results when borderline positive responses between SI values of 1.8 and 2.5 are obtained. For a weakly positive response which falls within this range, users may want to consider additional information, such as the strength of the dose-response relationship; evidence of systemic toxicity or excessive irritation; and where appropriate, statistical significance, together with the SI values, to confirm that such results are positive.

ICCVAM considers the applicability domain for the LLNA:DA to be the same as the traditional LLNA, unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA:DA. For example, the LLNA:DA may not be appropriate for testing substances which affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., ATP degrading enzymes or presence of extracellular ATP in the lymph node).

Following the Panel peer review meetings in 2008 and 2009, ICCVAM finalized its conclusions and recommendations for the LLNA:DA uses and limitations; the test protocol; the test method performance standards; and future studies. Based upon these activities, a draft test guideline for the LLNA:DA was submitted in July 2009, to the OECD member countries for review. CPSC staff and ICCVAM cohosted an OECD Expert Consultation meeting in October 2009, and a conference call in January 2010, to evaluate member country comments on the draft test guideline. A final draft test guideline was forwarded to the OECD to consider for adoption at their March 2010 meeting. The new test guideline, designated Test Guideline 442A, was adopted by the OECD in July 2010.

5. CPSC Staff Recommendation

Staff agrees with the ICCVAM recommendations on the LLNA:DA, with its utility in determining the sensitizing capacity of a substance. This modified form of the traditional LLNA also addresses animal welfare considerations, by providing nonradioactive methods that can reduce the number of animals needed per test, as well as result in less pain and suffering when used in place of the traditional guinea pig test methods for

countries that severely limit or discourage the use of radioactive materials, materials that are required by the traditional LLNA. Furthermore, under the current database, the stimulation index recommended for this test method avoids false negatives.

C. LLNA Applicability Domain

1. Background

The applicability domain refers to defined chemicals and products for which a test method can be used to obtain accurate and reliable results. In 2007, the CPSC requested that ICCVAM evaluate several modifications and applications of the traditional LLNA, including use of the LLNA to evaluate a broader range of substances. For the March 2008 Panel meeting, ICCVAM updated the original 1999 ICCVAM LLNA report, based upon a comprehensive review of data for more than 500 substances, an increase over the 209 reviewed in the original report. The Panel agreed with the ICCVAM recommendations that the LLNA appeared useful for testing of metal compounds, with the exception of nickel, and that more data were needed before definitive conclusions could be made for mixtures and substances in aqueous solutions. The Panel noted that the term *mixtures* was used too broadly, and this concern was addressed by dividing the substances into pesticide formulations, dyes, natural complex substances, and substances tested in aqueous solutions, and analyzing the data for each group separately. NICEATM continued to collect additional data. The Panel reconvened in April 2009, to review an updated draft Addendum, an evaluation on data derived from a database of more than 600 substances.

2. Validation and Performance

For this performance analysis, the ability of the LLNA to identify potential skin sensitizers was compared to human and guinea pig test data, if available, for each class of substances evaluated.

Pesticides: the database contained test results on 104 pesticide formulations, of which 23 had comparative guinea pig data. Human data was not available for these formulations to confirm their human sensitization potential. Based on these 23 formulations, the accuracy of the LLNA results compared to guinea pig data is 57 percent, with a false positive rate of 50 percent, and a false negative rate of 0 percent. Any formulation positive in the guinea pig test was positive in the LLNA.

Natural complex substances: the LLNA database contained test results for 12 natural complex substances with 75 percent classified as sensitizers and 25 percent as non-sensitizers. Based on this limited database, the accuracy of the LLNA results were 42percent compared to human clinical study data. The LLNA had a false positive rate of 75 percent and a false negative rate of 25 percent compared to human data. There was no comparative data from guinea pig tests.

Dyes: the database contained test results on six dyes that had comparative guinea pig data. Human data was not available for these formulations to confirm their human

sensitization potential. Based on this limited database, the accuracy of the LLNA results compared to guinea pig data is 33 percent, with a false positive rate of 100 percent (one substance) and a false negative rate of 60 percent.

Metal compounds: the database contained test results on 48 studies involving 16 metal compounds representing 13 different metals. All 16 compounds had comparative human data, and 8 had comparative guinea pig data. Due to conflicting data, nickel compounds were excluded from the metals performance analysis. The LLNA accuracy compared to human data is 86 percent, with a false positive rate of 40 percent, and a false negative rate of 0 percent. The accuracy of the LLNA results compared to guinea pig data is 83 percent, with a false positive rate of 100 percent (one substance), and a false negative rate of 0 percent. Note: the accuracy of guinea pig data on 6 substances compared to human data was 100 percent, with 0 percent false positive and false negative rates.

Substances in aqueous solutions: the database contained test results on 44 studies involving testing of 25 substances in aqueous solutions.[14] All 25 substances had comparative guinea pig data. Human data was available for only 1 substance, which was discordant between the LLNA and the guinea pig data. Based on these 25 substances, the LLNA accuracy compared to guinea pig data is 56 percent, with a false positive rate of 48 percent, and a false negative rate of 25 percent.

3. Recommendations for the Applicability Domain

The Panel reconvened in April 2009 to review and evaluate the revised Addendum and ICCVAM recommendations. The Panel provided final comments on the usefulness and limitations of the expanded applicability domain in their June 2009 report. The peer review panel concluded the following with regard to the expanded applicability domain:

- *Regarding the use of the LLNA for testing formulations, dyes, etc., the Panel acknowledged that the ability of ICCVAM to develop test method recommendations was limited not only by the amount of data available, but by the relatively poor concordance of traditional LLNA outcomes in comparison to those obtained in the guinea pig test." "The test materials for which data are provided in the revised draft Addendum cover only a subset of the active ingredients used in each of the relevant product classes." "The Panel noted that the revised draft Addendum does not consider many classes of formulations to which humans may be exposed, by intention or by accident, such as: metalworking fluids, fuels, petroleum products such as lubricants, detergents and other cleaning agents, enzymes used in cleaning products, chemical household products, chemical pharmaceutical products, medical device materials, and nanomaterials.*
- *The Panel recommends that Federal agencies considering the results of this validation process assess how representative the test materials and findings in the revised draft Addendum are relative to substances of interest.*
- *The Panel suggested that, unless there are unique physiochemical properties associated with a material that might affect its ability to interact with the immune*

[14] An aqueous solution was defined by ICCVAM as substances containing at least 20 percent water.

process (e.g., nanomaterials), it should be a candidate for LLNA testing. The Panel disagreed with the revised draft ICCVAM recommendation that a definitive recommendation on the usefulness of the LLNA for testing natural complex substances and dyes could not be made until more data were accrued. The Panel considered these classes of materials suitable for testing in the LLNA.

- *The Panel agreed with the revised draft ICCVAM recommendations noting that the high rate of false positive substances may be inherent to the product and/or chemical class, testing of substances at concentrations that produced skin irritation, and to the fact that the LLNA detects the induction phase of skin sensitization.*
- *The Panel concluded that the updated information on various elements in the Addendum did not suggest the need for changes to recommendations for the development of a revised standard method. Whenever discretion is permitted, the Panel recommended the inclusion of a suitable (representative) positive control from the same category of materials to be tested.*

ICCVAM recommended the following future studies to further characterize the usefulness and limitations of the LLNA; however, ICCVAM discourages formal validation of the LLNA for new classes of test substances unless there is a biologically-based rationale:

- To more comprehensively evaluate the effectiveness of the LLNA for testing nickel compounds, additional LLNA data on such compounds with comparative human or guinea pig data is needed.
- If available, solubility data should be provided in future studies.
- For new classes of test materials, an integrated assessment of available information should be conducted. This should include computer-assisted structure activity relationships (QSAR); prediction/measurement of biotransformation to potential reactive species; and possibly peptide, protein, or lipid binding. Before animal testing is conducted, consideration should be given to the necessity for a substance to be tested for skin sensitization potential.
- If any variant of the LLNA is validated for use to test novel classes, then the findings should be relevant to the family of validated LLNA tests.

4. ICCVAM Conclusion

ICCVAM recommended that, although the database is limited, the LLNA may be used to test any chemical or product, including pesticide formulations, metals, substances in aqueous solutions, and other products, such as natural complex substances and dyes, unless the chemical or product to be tested has properties that may interfere with the ability of the LLNA to detect skin-sensitizing substances. To achieve adequate exposure, substances in aqueous solutions must be tested in an appropriate vehicle that will maintain adequate contact of the test substance with the skin.

The LLNA is more likely to yield a positive result for pesticides, dyes, metals, and aqueous solutions than the guinea pig test. The potential for over-classification may be a limitation of the LLNA; however, the false negative rate was 0 percent for metal compounds and pesticide formulations.

Following the Panel peer review meetings in 2008 and 2009, ICCVAM finalized its conclusions and recommendations for the expanded applicability domain. Based upon these activities, a draft revision to TG429, the test guideline for the traditional LLNA, was submitted in July 2009 to the OECD member countries for review. CPSC staff and ICCVAM cohosted an OECD Expert Consultation meeting in October 2009 and a conference call in January 2010, to evaluate member country comments on the draft test guideline. The revised test guideline was adopted by the OECD in July 2010, incorporating the updated LLNA applicability domain.

5. CPSC Staff Recommendation

Staff recommends acceptance of the expanded applicability domain, which strengthens the existing LLNA test guideline and provides further support for the development of improved versions of the method, as well as provides consistency in utilization of the assay, an assay which will be used as the gold standard for validation of alternative *in vitro, in silico,* or *in vivo* methods for determining sensitization. The database, although large, is limited to product classes, and staff would encourage the continuation of data collection for different product classes/formulations to which the consumer can be exposed.

III. Related Events Regarding Sensitizer Testing

The GHS (Globally Harmonized System) is an internationally harmonized approach to classification and labeling for all chemicals, and mixtures of chemicals. The CPSC is a member of the U.S. federal interagency work group participating in the development and possible implementation of the GHS. The issue of sensitizers is addressed by the GHS in chapter 3.4. Health Sciences (HS) staff is part of an OECD expert group that was formed to develop a revised GHS approach on these issues.

In March, 2008, the OECD sensitization expert group met at the CPSC to continue work on a proposal for revising the GHS chapter for skin sensitizers with respect to strong versus weak sensitizers (GHS chapter 3.4 addresses both respiratory and skin sensitizers). At its April 2008 meeting, the OECD Task Force on Harmonisation of Classification and Labelling agreed to the proposed revisions. The revised sensitizer chapter was submitted to the UN Sub-Committee of Experts on the GHS as a formal proposal and was accepted.

Health Sciences staff believes that the proposed GHS approach for classifying and labeling chemicals that are sensitizers generally will be compatible with the FHSA "strong sensitizer" statutory and supplemental definition.

One of the issues that arose from discussions with the OECD expert group was that of sensitizer potency and tests that can be used to determine potency of chemicals that might be sensitizers. European scientists favored the sole use of the LLNA for the determination of sensitizer potency. The criteria recently adopted by the GHS to distinguish strong sensitizers from other sensitizers, is based on human, guinea pig, and LLNA data. Substances with positive responses in the human maximization test (HMT) or human repeat insult patch test (HRIPT) at induction

thresholds ≤500 μg/cm^2 are classified as strong sensitizers. Similarly, LLNA EC3 values ≤ 2% are proposed to categorize substances as strong sensitizers and LLNA EC3 values >2 to categorize substances as "other sensitizers." Because of concerns about the scientific validity of this approach, CPSC staff nominated the LLNA test method, for determination of sensitization potency, to ICCVAM for its review. ICCAM was requested, in particular, to review the validation status of the use of the LLNA as a standalone assay for the determination of potency. In order to evaluate the accuracy of the LLNA for identifying strong sensitizers as defined by human data, NICEATM and ICCVAM used a database of 112 substances with both LLNA and human data to calculate human potency classification categories (strong vs. other than strong). In March 2008, the ICCVAM Peer Review Panel recommended that the LLNA should be used as part of a weight-of-evidence approach for potency determinations, not as a standalone assay. As a result, CPSC staff was able to persuade its European counterparts on the OECD expert panel to agree that the revisions to the GHS sensitization chapter embrace the use of the LLNA as part of a weight-of-evidence approach, not as a standalone test.

IV. ICCVAM Recommendations

ICCVAM concluded that the scientific validity of the BrdU-ELISA and the LLNA:DA has been evaluated adequately and supports the use of both test methods to distinguish between skin sensitizers and nonsensitizers. ICCVAM recommends that stimulation indices of ≥ 1.6 and ≥ 1.8 be used as the decision criteria for the BrdU-ELISA and the LLNA:DA, respectively, to identify substances as potential sensitizers. This recommendation is based on the fact that no false negatives occurred when these SI values were used. There is a possibility of false positive results for both methods. ICCVAM recommended that, although the database is limited, the LLNA may be used to test any chemical or product, including pesticide formulations, metals, substances in aqueous solutions, and other products, such as natural complex substances and dyes, unless the chemical or product to be tested has properties that may interfere with the ability of the LLNA to detect skin-sensitizing substances.

Following the Panel meetings, ICCVAM finalized its conclusions and recommendations for the BrdU-ELISA, the LLNA:DA, and the expanded applicability domain. Based upon these activities, a draft revision to TG429, the test guideline for the traditional LLNA, as well as a draft test guidelines for the BrdU-ELISA and the LLNA:DA were submitted in July 2009, to the OECD member countries for review. CPSC staff and ICCVAM cohosted an OECD Expert Consultation meeting in October 2009, and a conference call in January 2010, to evaluate member country comments on the draft test guidelines. The revised test guideline incorporating the updated LLNA applicability domain, as well as the test guidelines for the BrdU-ELISA and the LLNA:DA were adopted by the OECD in July 2010. The new test guidelines for the nonradioactive versions of the LLNA are designated as Test Guideline 442A and 442B.

V. Discussion by CPSC Staff

Staff agrees with the ICCVAM recommendations on the nonradioactive versions of the LLNA, the BrdU-ELISA, and the LLNA:DA, with their utility in determining the sensitizing capacity of a substance. These modified forms of the traditional LLNA also address animal welfare considerations by providing nonradioactive methods that can reduce the number of animals needed per test, as well as result in less pain and suffering when used in place of the traditional

guinea pig test methods for countries that severely limit or discourage the use of radioactive materials, materials which are required by the traditional LLNA. Furthermore, under the current database, the stimulation indices recommended for both nonradioactive methods avoid false negatives. The OECD Expert Group suggested that these methods will provide adequate (and perhaps even improved) protection of human health.

Some concerns had been voiced by OECD Expert Group participants regarding the use of BrdU since it is classified in Europe as "harmful" and with "suspected carcinogen potential." However, the conclusion of the OECD Expert Group was that BrdU has been routinely used for a wide range of procedures over many years and workers can take the appropriate precautions when handling the substance.

As mentioned in the background section for the LLNA:DA, the Panel viewed the treatment schedule difference between the LLNA:DA and the traditional LLNA potentially to be significant if the LLNA:DA induced the elicitation phase of skin sensitization. The Panel was also concerned that the 1 percent sodium lauryl sulfate (SLS) pretreatment step might modify the inherent sensitivity of the LLNA. The Panel was concerned if the duration of the test encompassed the induction and elicitation phases of allergic contact dermatitis; this encroachment into the elicitation phase could lead to some loss of the animal welfare benefits that the LLNA provides over guinea pig tests. Through discussions among the OECD Expert Group, it was concluded that although there was speculation that the dosing regimen may extend the induction phase, there has been no evidence to suggest that animal welfare is compromised by signs of the elicitation phase (e.g., no evidence of ear swelling or redness on Days 7 and 8). Unpublished data also demonstrates that it typically takes at least two weeks, a duration of time not approached by the LLNA:DA test method, for an elicitation reaction to manifest using the LLNA protocol. The SLS pretreatment in the LLNA:DA test method consists of application of a 1 percent aqueous solution. This concentration of SLS does not induce excessive irritation, nor is it considered a sensitizing dose based on traditional LLNA data. SLS is an irritant in mice at doses starting at 10 percent in dimethylformamide. A question was raised during the meeting with the OECD Expert Group regarding degradation of ATP, the marker used by the LLNA:DA method to assess lymphocyte cell proliferation during the length of the assay. The LLNA:DA protocol contains specific elements designed to minimize inaccuracies caused by biodegradation of ATP in lymph node preparations. Thus, it was concluded by the OECD Expert Group that the LLNA:DA provides a valid nonradioactive alternative to guinea pig tests.

Even though the LLNA has been tested with a variety of mixtures, the issue of whether an appropriate representative set of mixtures has been tested was discussed among the OECD Expert Group. The meeting participants agreed that this is a complicated issue, mainly due to the inherent problems of testing of mixtures and formulations, and it applies across all toxicity test methods, not just the LLNA. Since the LLNA test guideline (TG429) already includes reference to benchmark controls, the meeting participants agreed that their usefulness should be emphasized in the context of testing new mixtures/formulations. CSPC staff agrees with both the Panel's and ICCVAM's recommendation to continue to accrue data, because the revised draft Addendum does not consider many classes of formulations to which humans may be exposed, particularly classes of substances that may fall under CPSC's jurisdiction.

In 1984, the Commission adopted a policy to reduce the number of animals tested and to minimize the pain and suffering associated with testing (49 FR 22522). In addition, the utilization of laboratory animals is recommended in a tiered and sequential approach to testing. In a tiered-testing strategy, the test substance is tested *in vivo* if the appropriate hazard determination cannot be made from physicochemical characteristics, expert opinion, prior human experience, or animal testing. Under the FHSA, the determination of whether a substance is a "strong sensitizer" is based upon a weight-of-evidence approach. In the FHSA supplemental "strong sensitizer" definition, it is written:

> *Strong: In determining that a substance is a "strong" sensitizer, the Commission shall consider the available data for a number of factors. These factors should include any or all of the following (if available):*
> - *Quantitative or qualitative risk assessment*
> - *Frequency of occurrence and range of severity of reactions in healthy or susceptible populations*
> - *The result of experimental assays in animals or humans (considering dose-response factors), with human data taking precedence over animal data*
> - *Other data on potency or bioavailability of sensitizers*
> - *Data on reactions to a cross-reacting substance or to a chemical that metabolizes or degrades to form the same or a cross-reacting substance*
> - *The threshold of human sensitivity*
> - *Epidemiological studies*
> - *Case histories*
> - *Occupational studies*
> - *Other appropriate in vivo and in vitro test studies*

Therefore, the nonradioactive versions of the LLNA would fit into a weight-of-evidence evaluation under the FHSA. Staff believes that the draft test method recommendations for the BrdU-ELISA and the LLNA:DA adequately addressed any false positive results by giving cautionary and weight-of-evidence consideration to the positive substances.

Staff agrees with ICCVAM that the two nonradioactive versions of the LLNA, the BrdU-ELISA and the LLNA:DA, and the expanded LLNA applicability domain, are based on sound science and are valid scientifically for their proposed uses.

VI. Recommendations by CPSC Staff

Staff recommends accepting the ICCVAM recommendations. Thus, staff recommends acceptance of the internationally harmonized nonradioactive BrdU-ELISA and LLNA:DA methods for hazard identification of substances that could be sensitizers. Staff also recommends acceptance of the expanded applicability domain that strengthens the existing LLNA test guideline and provides further support for the development of improved versions of the method, as well as provides consistency in utilization of the assay, an assay which will be used as the gold standard for validation of alternative *in vitro, in silico,* or *in vivo* methods for determining sensitization.

Labeling of a consumer product regarding the hazards associated with that product is required by the FHSA. In order to determine the appropriate cautionary labeling for "strong sensitizers," animal testing may be necessary. However, the Commission supports minimizing the number of animals used and reducing the pain or suffering associated with animal testing and encourages the development and use of alternatives to animal test models. Thus, the staff recommends that the Commission accept the ICCVAM recommendations because these alternative nonradioactive LLNA test method protocols encourage the reduction, refinement, or replacement of animals in testing and the data indicate that the methods are scientifically valid methods. Further, the FHSA requires a weight-of-evidence approach. In this context, these alternative LLNA methods and the expanded applicability domain may result in additional data that could be used to make a determination if an undiluted chemical or a mixture is a "strong sensitizer."

Staff will draft a letter to ICCVAM indicating the Commission's actions with regard to the ICCVAM recommendations. The ICCVAM website (http://iccvam.niehs.nih.gov/home.htm) will link to the Commission website where we will post our acceptance or nonacceptance of the three recommendations. In the section of the ICCVAM website, Pertinent Regulations, Guidelines and Laws (http://iccvam.niehs.nih.gov/agencies/regs.htm), there will be an announcement of the Commission's action on the acceptance or nonacceptance of the three ICCVAM recommendations. Once ICCVAM receives responses from all the agencies, it will publish a Federal Register notice announcing all the agencies' responses.

VII. Options

The Commission can vote to:

1. Accept the ICCVAM recommendations and instruct staff to draft a letter to ICCVAM indicating acceptance of its recommendations.

2. Reject the ICCVAM recommendations and instruct staff to draft a letter to ICCVAM indicating rejection of its recommendations.

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