Draft ICCVAM Test Method Recommendations
Non-Radioactive LLNA: DA

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This document provides draft ICCVAM recommendations on the non-radioactive LLNA: DA, a test method for assessing the allergic contact dermatitis potential of chemicals and products for regulatory testing. These draft recommendations are based on information and data provided in a draft background review document available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm, and will be considered by an independent scientific peer review panel that will meet in public session on April 28–29, 2009. Public comments are welcome. More information is available in the *Federal Register* notice of the meeting (74 FR 8974) available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-4280.pdf. ICCVAM will finalize these recommendations after consideration of comments from the peer review panel, the public, and its scientific advisory committee.

These draft recommendations do not represent the official position of any Federal agency.

1.0 Draft Recommendations: Test Method Uses and Limitations

Background

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is currently evaluating the validation status of the LLNA: DA as a non-radioactive modification of the traditional LLNA (i.e., ICCVAM 1999; Dean et al. 2001) to identify substances that may cause allergic contact dermatitis (ACD). While the traditional LLNA assesses cellular proliferation by measuring the incorporation of radioactive thymidine into the deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: DA assesses cell proliferation by measuring the level of adenosine triphosphate (ATP) in the auricular lymph nodes. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule and the addition of pretreatment of the application site with a nonirritating concentration of sodium lauryl sulfate (SLS) (see **Section 2.0** of the draft ICCVAM LLNA: DA Background Review Document [ICCVAM 2009]). A comprehensive report on the data and information supporting the validity of this test method, including its accuracy and reliability compared to the traditional LLNA, is also provided in the draft ICCVAM LLNA: DA Background Review Document (ICCVAM 2009).

ICCVAM has developed recommended test method performance standards for the LLNA (ICCVAM 2009), [1] which are proposed to evaluate the performance of modified LLNA test methods that are mechanistically and functionally similar to the traditional LLNA. However, because the validation studies for the LLNA: DA test method were completed prior to the development of LLNA performance standards, and some of the protocol modifications noted above may be considered functionally and mechanistically different from the traditional LLNA (i.e., pretreatment with SLS, extended dosing schedule), the ICCVAM LLNA performance standards were not used to evaluate the LLNA: DA.

[1] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.

Draft Recommendations

Based on the available validation database of 44 substances with sufficient traditional LLNA data (32 sensitizers and 12 nonsensitizers), ICCVAM proposes that the accuracy and reliability of the LLNA: DA supports the use of the test method to identify substances as potential skin sensitizers and nonsensitizers, with specific defined limitations. ICCVAM proposes that a decision criterion of stimulation index (SI) ≥ 2.5 be used to identify potential sensitizers. ICCVAM bases this proposal on the fact that no false positives, relative to the traditional LLNA, resulted when an SI ≥ 2.5 was obtained. (When an SI ≥ 2.5 was obtained in the LLNA: DA, the false positive rate compared to the traditional LLNA was 0% [0/12]).[2]) ICCVAM proposes that a decision criterion of SI ≤ 1.7 be used to identify nonsensitizers based on the fact that no false negatives, relative to the traditional LLNA, resulted when an SI ≤ 1.7 was obtained (when an SI ≤ 1.7 was obtained in the LLNA: DA, the false negative rate compared to the traditional LLNA was 0% [0/32]).

However, when an SI between 1.7 and 2.5 is obtained in the LLNA: DA (the SI range for 5 LLNA positives and 5 LLNA negatives),[3] users should carefully consider the interpretation of LLNA: DA results in an integrated decision strategy in conjunction with all other available information (e.g., dose response information, statistical analyses of treated vs. control animals, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is sufficient information with which to base an accurate determination of sensitization potential, or if additional testing is necessary.

As an example, consider an LLNA: DA result of SI = 2.2 coupled with (1) a low molecular weight (e.g., < 300) such that the substance could easily traverse the stratum corneum; (2) evidence that the substance is moderately peptide reactive; (3) a statistically significant difference between treated and vehicle control animals; and (4) a clear dose response. While none of this information alone might be considered adequate to reach a conclusion, all of the information together might be considered sufficient to classify this substance as a potential sensitizer. Such an integrated decision strategy would need to be conducted on a case-by-case basis.

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[2] For the accuracy analyses, results for substances tested multiple times were combined so that each substance was represented by one result. In this case, the single result used for each substance represented the most prevalent outcome. Multiple tests were available for 14 substances tested with the LLNA: DA.

[3] Within the validation database for the LLNA: DA, 10 substances produced maximum SI values between 1.7 and 2.5. Among these 10 substances, 5/10 are sensitizers and 5/10 are nonsensitizers based on traditional LLNA results.

Limitations

As described above, LLNA: DA results are increasingly uncertain, compared to the traditional LLNA, when the SI is between 1.7 and 2.5. Accordingly, additional information and/or testing must be considered in order to reach a classification decision for such results.

In addition, inconsistent results for nickel sulfate in the validation study suggest that the LLNA: DA may not be suitable for testing substances containing nickel. Until the LLNA: DA has been found to accurately identify ACD potential in substances containing nickel, further testing using a different test system is recommended when negative results are obtained for such substances.

2.0 Draft Recommendations: Test Method Protocol for the LLNA: DA

The ICCVAM-recommended LLNA: DA test method protocol is based on the protocol developed by Idehara et al. (2008; see **Appendix A** of the draft ICCVAM LLNA: DA Background Review Document). The ICCVAM-recommended LLNA: DA test method protocol incorporates all aspects of the recently recommended ICCVAM LLNA test method protocol (ICCVAM 2009) except for those procedures unique to the LLNA: DA (see **Appendix A** of the draft background review document). Key aspects included in the ICCVAM-recommended protocol include the following:

- The high dose should be the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation.
- A minimum of four animals per dose group is recommended.
- Collection of individual animal data is recommended.
- Inclusion of a concurrent vehicle control and positive control in each study is recommended.

Additionally, ICCVAM recommends there should be a measure of variability of the positive control response over time. Laboratories should maintain a historical database of positive control SI values such that results can be compared to the mean historical SI. There could be cause for concern when a negative test substance result is accompanied by a concurrent positive control SI value significantly lower than the mean historical SI.

In testing situations where dose-response information is not required, the LLNA: DA should be considered for use as a reduced LLNA protocol, further reducing animal use.

3.0 Draft Recommendations: Future Studies

- Efforts should be made to further characterize the sensitization potential of substances that produce an SI between 1.7 and 2.5 in the LLNA: DA. This could include evaluations of peptide-binding activity, determination of molecular weight, identification of results from related chemicals, or consideration of other testing data (e.g., *in vitro* results).
- Inconsistent results for nickel sulfate suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, consistent with recommendations for the traditional LLNA, additional data from LLNA: DA studies on such compounds with comparative human and/or guinea pig data are needed in order to more comprehensively evaluate the suitability of the LLNA: DA for testing of nickel compounds.
- Additional skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: DA.
- Efforts should be made to identify additional human data and human experience for test substances. This data may be used to further assess the usefulness and limitations of this and other versions of the LLNA for identifying human-sensitizing substances (e.g., formulations). Such efforts might include post-marketing surveillance of consumers for allergic reactions and occupational surveillance of potentially exposed workers.
- Draft Performance Standards

ICCVAM has developed performance standards for the traditional LLNA (ICCVAM 2009) to evaluate the performance of LLNA test methods that incorporate specific protocol modifications (e.g., procedures to measure lymphocyte proliferation) compared to the traditional LLNA. However, as noted above, these performance standards are not applicable to the LLNA: DA, because it incorporates procedures that differ slightly from the essential test method components in the performance standards for the traditional LLNA. Accordingly, ICCVAM will develop performance standards for the LLNA: DA that can be used to evaluate future modifications of the LLNA: DA.

4.0 References

Dean JH, Twerdok LE, Tice RR, Sailstad DM, Hattan DG, Stokes WS. 2001. ICCVAM evaluation of the murine local lymph node assay. Conclusions and recommendations of an independent scientific peer review panel. Regul Toxicol Pharmacol 34:258–273.

ICCVAM. 1999. The murine local lymph node assay: A test method for assessing the allergic contact dermatitis potential of chemical/compounds. NIH Publication No. 99-4494. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

ICCVAM. 2009. Recommended Performance Standards: Murine Local Lymph Node Assay. NIH Publication Number 09-7357. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

ICCVAM. 2009. Revised Draft Background Review Document – Non-radioactive Murine Local Lymph Node Assay. Modified by Daicel Chemical Industries, Ltd. Based on ATP Content Test Method Protocol (LLNA: DA).

Idehara K, Yamagishi G, Yamashita K, Ito M. J. 2008. Characterization and evaluation of a modified local lymph node assay using ATP content as a non-radio isotopic endpoint. Pharmacol Toxicol Methods 58:1–10.