Draft ICCVAM Test Method Recommendations
Non-Radioactive LLNA: BrdU-ELISA

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This document provides draft ICCVAM recommendations on the non-radioactive LLNA: BrdU-ELISA, 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 murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (LLNA: BrdU-ELISA) as a non-radioactive modification of the traditional murine local lymph node assay (LLNA; i.e., ICCVAM 1999; Dean et al. 2001) to identify substances that may cause allergic contact dermatitis (ACD) for regulatory hazard classification and labeling purposes. While the traditional LLNA assesses cellular proliferation by measuring the incorporation of radioactive tritiated thymidine into the deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: BrdU-ELISA assesses the same endpoint by measuring the incorporation of the thymidine analog bromodeoxyuridine (BrdU) using an enzyme-linked immunosorbent assay (ELISA). A comprehensive evaluation of this test method, including its accuracy and reliability compared to the traditional LLNA, is provided in the revised draft ICCVAM LLNA: BrdU-ELISA Background Review Document (BRD, 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: BrdU-ELISA test method were completed prior to the development of LLNA performance standards and because data for all of the performance standards reference substances were not available, the ICCVAM LLNA performance standards were not used as the basis for evaluating the validity of the LLNA: BrdU-ELISA.

Draft Recommendations

Based on the available validation database of 31 substances (22 sensitizers and 9 nonsensitizers), ICCVAM proposes that the accuracy and reliability of the LLNA: BrdU-ELISA 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 a stimulation index (SI) ≥ 2.0 be used to identify potential sensitizers, based on the fact that no false positives relative to the traditional LLNA resulted when an SI ≥ 2.0 was

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

obtained (when an SI ≥ 2.0 was obtained in the LLNA: BrdU-ELISA, the false positive rate compared to the traditional LLNA is 0% [0/9])[2]. Likewise, ICCVAM proposes that a decision criterion of SI < 1.3 be used to identify nonsensitizers, based on the fact that no false negatives relative to the traditional LLNA resulted when an SI < 1.3 was obtained (when an SI < 1.3 was obtained in the LLNA: BrdU-ELISA, the false negative rate compared to the traditional LLNA is 0% [0/22]).

However, six traditional LLNA positives and five traditional LLNA negatives produced an SI within the range of 1.3 to <2.0 in the LLNA: BrdU-ELISA[3]. Therefore, when results are obtained in this range, users should carefully consider the interpretation of LLNA: BrdU-ELISA 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 on which to determine sensitization potential, or if additional testing is necessary.

As an example, consider an LLNA: BrdU-ELISA result of SI = 1.8, 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 any of this information alone would not necessarily be considered adequate to reach a conclusion, all of the information together might be considered sufficient to consider this substance a potential sensitizer. Such an integrated decision would need to be conducted on a case-by-case basis.

Limitations

As discussed above, when an SI greater than or equal to 1.3 but less than 2.0 is obtained, there is increased uncertainty as to whether the substance is a sensitizer or a non-sensitizer, and additional information or testing must be considered and used to reach a hazard classification decision.

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

[3] Within the validation database for the LLNA: BrdU-ELISA, 11 substances produced an SI of 1.3 to < 2.0. Among these 11 substances, 6/11 are sensitizers and 5/11 are nonsensitizers based on traditional LLNA results.

2.0 Draft Recommendations: Test Method Protocol for the LLNA: BrdU-ELISA

The draft ICCVAM-recommended LLNA: BrdU-ELISA is based on the protocol developed by Takeyoshi et al. (2004, see Appendix A of the draft ICCVAM LLNA: BrdU-ELISA BRD). The draft ICCVAM-recommended LLNA: BrdU-ELISA protocol incorporates all aspects of the recently updated ICCVAM recommended LLNA test method protocol (Appendix A of ICCVAM 2009), except for those procedures unique to the conduct of the LLNA: BrdU-ELISA (see Appendix A of the draft BRD). Key aspects that are included in the ICCVAM-recommended protocol include the following:

- The high dose group 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 that is significantly lower than the mean historical SI.

3.0 Draft Recommendations: Future Studies

- Efforts should be made to further characterize the sensitization potential of substances that produce an SI of 1.3 to less than 2.0 in the LLNA: BrdU-ELISA. This could include evaluations of peptide binding activity, determination of molecular weight, identifying results from related chemicals, human studies where ethical, review of occupational exposures and postmarketing experience or monitoring, or other testing data (e.g., *in vitro* results).
- Consistent with recommendations for the traditional LLNA, to more comprehensively evaluate the ability of the LLNA: BrdU-ELISA to be used for testing metal compounds, additional data from LLNA: BrdU-ELISA studies on such compounds with comparative human and/or guinea pig data are needed.

- Additional skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: BrdU-ELISA.
- Efforts should be made to identify additional human data and human experience for test substances that can 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).

4.0 Draft Performance Standards

Unique performance standards for the LLNA: BrdU-ELISA are not proposed at this time. Because the LLNA: BrdU-ELISA is mechanistically and functionally similar to the traditional LLNA, ICCVAM proposes that the ICCVAM LLNA performance standards (ICCVAM 2009) can be used to evaluate future modifications of the LLNA: BrdU-ELISA.

5.0 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: BrdU-ELISA Test Method Protocol (LLNA: BrdU-ELISA). Available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm.

Takeyoshi M, Noda S, Yamazaki S, Kakishima H, Yamasaki K, Kimber I. 2004. Assessment of the skin sensitization potency of eugenol and its dimers using a non-radioisotopic modification of the local lymph node assay. J Appl Toxicol 24:77-81.