Draft ICCVAM Test Method Recommendations:

Non-Radioactive LLNA: BrdU-FC

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1.0 Draft Recommendations: Test Method Uses and Limitations

Background: ICCVAM is currently evaluating the validation status of the LLNA: BrdU-FC as a non-radioactive alternative to 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 radioactivity into the deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: BrdU-FC assesses the same endpoint by measuring the incorporation of the thymidine analog bromodeoxyuridine (BrdU). The incorporation of BrdU is measured using flow cytometry. The LLNA: BrdU-FC also includes a routine assessment of ear swelling as a measure of excessive irritation. Finally, the LLNA: BrdU-FC also includes enhancements (referred to hereafter as the "eLLNA: BrdU-FC"), for substances with a stimulation index (SI) ≥ 3 and with ear swelling > 25% that include assessment of immunophenotypic markers to distinguish sensitizers from irritants (see **Section 2.0** of the draft LLNA: BrdU-FC BRD). Additional information and discussion of the evaluation of this test method are provided in the draft ICCVAM LLNA: BrdU-FC BRD (ICCVAM 2007).

Draft Recommendations:

Based on the available database of 45 substances (26 sensitizers and 19 nonsensitizers when tested in the traditional LLNA) and demonstrated performance (accuracy of 91% [41/45], sensitivity of 100% [26/26], specificity of 79% [15/19]) compared to the traditional LLNA, the LLNA: BrdU-FC may useful for identifying substances as potential skin sensitizers and non-sensitizers. However, at this time, more information and data are needed before a recommended use of the LLNA: BrdU-FC can be made. Specifically:

- Three of the 45 substances produced an "equivocal" result[1] in the LLNA: BrdU-FC, one of which has been commonly used as a positive control in the traditional LLNA (2-mercaptobenzthiazole). One of the other equivocal substances is one of the recommended reference standard sensitizers in the draft ICCVAM LLNA

[1] Equivocal is defined by MB Research Labs as multiple tests with the same substance that produce discordant results.

performance standards; in order for an assay like the LLNA: BrdU-FC to demonstrate equivalence to the traditional LLNA, these draft performance standards require complete concordance with the recommended sensitizers and nonsensitizers. The rationale for the repeat testing of these substances and possible reasons for the discordant results have been requested, but have not been provided.

- There has not been an evaluation of interlaboratory reproducibility. This is critical if this test method is to be accepted for use in laboratories other than that of the test method developer.
- Original records have not been obtained for the studies included in this evaluation. As a result, an independent audit could not be conducted to confirm that the reported data is the same as the data originally recorded.

2.0 Draft Recommendations: Test Method Protocol for the LLNA: BRDU-FC

All aspects of the recommended ICCVAM LLNA test method protocol (ICCVAM 1999, Dean et al. 2001) should be followed with the exception of the method used to assess lymphocyte proliferation. Measurement of BrdU incorporation and total lymph node cell number using flow cytometry (i.e., total number of lymphocytes in the test preparation and the total number of cells with incorporated BrdU) is described in **Appendix A** of the draft ICCVAM LLNA: BrdU-FC BRD. In addition, an assessment of ear swelling should be routinely conducted to identify local irritation.

3.0 Draft Recommendations: Future Studies

- Further development of this test method is required to ensure that at least two of the three equivocal substances provide reproducible results. This is needed to meet the requirements of the draft ICCVAM LLNA performance standards that mandate, in order for this test method to demonstrate equivalence to the traditional LLNA, 100% concordance with the calls obtained in the traditional LLNA.
- The “sequential strategy” used in the enhanced FC-LLNA protocol, which incorporates immunophenotypic endpoints via flow cytometry to identify

potential false positives that are actually non-sensitizing skin irritants, should be further investigated to more fully characterize its usefulness and limitations for this purpose. Also, a thorough description of the immunochemical phenotyping procedure (e.g., type and source of antibody) should be provided.

- Prior to the use of this test method in other laboratories, the reference substances (HCA and DNCB) recommended for intra- and inter-laboratory reproducibility assessments in the ICCVAM draft performance standards should be tested to determine if acceptable results can be obtained.
- The applicability of the LLNA: BrdU-FC to testing metals, mixtures, and aqueous solutions (current limitations of the traditional LLNA) should be further evaluated to determine if this method can be used to assess the ACD potential of these types of substances.

4.0 Draft Performance Standards

Performance standards for the LLNA: BrdU-FC are not proposed at this time. However, ICCVAM has developed draft performance standards for the traditional LLNA (http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). These draft test method performance standards are proposed to evaluate the performance of LLNA test methods that incorporate specific protocol modifications to measure lymphocyte proliferation compared to the traditional LLNA. ICCVAM does not anticipate the need to develop separate performance standards for the LLNA: BrdU-FC.