1.0 Introduction and Rationale for the Proposed Use of the Reduced Murine Local Lymph Node Assay (rLLNA) to Identify Skin Sensitizers

1.1 Introduction

1.1.1 Historical Background

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine local lymph node assay (traditional LLNA[35]) as a valid substitute for currently accepted guinea pig test methods to assess allergic contact dermatitis (ACD) potential of most types of substances. ICCVAM based its recommendation on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the NICEATM–ICCVAM website.[36]

ICCVAM forwarded to U.S. Federal agencies its recommendation that the traditional LLNA should be considered for regulatory acceptance or other non-regulatory applications for assessing the ACD potential of substances, while recognizing that some testing situations would still require the use of traditional guinea pig test methods (ICCVAM 1999). The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (International Organization for Standardization [ISO] 10993-10: Tests for Irritation and Sensitization [ISO 2002]; Organisation for Economic Co-operation and Development Test Guideline [TG] 429 [OECD 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test Guideline OPPTS 870.2600: Skin Sensitization [EPA 2003]).

1.1.2 Allergic Contact Dermatitis

ACD is a frequent occupational health problem. According to the U.S. Department of Labor Bureau of Labor Statistics, in 2005, 980 cases of ACD involved days away from work.[37]

ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. Induction depends on the substance passing through the epidermis, where it forms a hapten complex with dermal proteins. Langerhans cells, the resident antigen-presenting cells in the skin, process the hapten complex. The processed hapten complex then migrates to the draining lymph nodes. Antigen presentation to T-lymphocytes follows, which leads to the clonal expansion of these cells. At this point, the individual is sensitized to the substance (Basketter et al. 2003; Jowsey et al. 2006). Studies have shown that the magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops (Kimber and Dearman 1991, 1996).

During the elicitation phase, the individual is again topically exposed to the substance. As in the induction phase, the substance penetrates the epidermis, is processed by the Langerhans cells, and is presented to circulating T-lymphocytes. The T-lymphocytes are then activated, which

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[35] The "traditional LLNA" refers to the validated ICCVAM-recommended LLNA (ICCVAM 1999), which measures lymphocyte proliferation based on incorporation of tritiated thymidine into the cells of the draining auricular lymph nodes.

[36] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf

[37] Available at http://www.bls.gov/IIF.

causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Basketter et al. 2003; Jowsey et al. 2006).

1.1.3 *U.S. Consumer Product Safety Commission (CPSC) Nomination*

On January 10, 2007, the CPSC formally requested that ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) evaluate several activities related to the LLNA.[38] The nominated activities included the following:

- The LLNA as a stand-alone assay for potency determination (including severity) for classification purposes

- Non-radioactive LLNA protocols

- The reduced LLNA (rLLNA) (also known as the ''cut-down'' or ''limit dose'' LLNA procedure)

- The use of the LLNA to test mixtures, aqueous solutions, and metals

ICCVAM unanimously agreed that the nominated activities should have a high priority for evaluation. ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), also recommended that the nominated activities be undertaken with a high priority.

As ICCVAM and NICEATM collaborate closely with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods, both organizations identified liaisons to the ICCVAM Immunotoxicity Working Group to facilitate the evaluations requested by the CPSC.

1.1.4 *Description of the Reduced Murine Local Lymph Node Assay*

Kimber and colleagues initially discussed the rLLNA in a 2006 publication (Kimber et al. 2006). The rLLNA was also discussed in two posters (Basketter et al. 2007; Chaney et al. 2007, subsequently published as Ryan et al. 2008) and one platform presentation (Basketter 2007) at the Society of Toxicology (SOT) Annual Meeting in Charlotte, NC, on March 25–29, 2007.

The protocol for the rLLNA is identical to that of the traditional LLNA (as described in the 1999 ICCVAM-recommended protocol) with one exception. In the traditional LLNA, three dose levels of each test substance are tested, while in the rLLNA only the highest dose level that does not induce systemic toxicity and/or excessive skin irritation is tested for skin-sensitizing activity (Kimber et al. 2006).

The term "limit dose," sometimes used to refer to the rLLNA, accurately depicts a modified LLNA that tests only the highest dose level that does not induce local irritation and/or systemic toxicity. The terms ''cut-down'' and ''reduced'' LLNA also accurately describe the reduction in the number of doses tested and emphasize the reduction in the number of animals used to perform the test. For consistency with the terminology presented in the publications that first described this version of the LLNA, the term "reduced LLNA" (rLLNA) will be used.

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[38] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

1.1.5   Results of an ECVAM Peer Review of the rLLNA

The ECVAM Scientific Advisory Committee (ESAC) established a review panel to retrospectively analyze the published LLNA data to determine if limiting the number of test substance dose levels to only the highest dose level could successfully reduce the number of animals used per test. The review was based on the evaluation published by Kimber et al. (2006). At its semi-annual meeting on April 26–27, 2007, ESAC reviewed the rLLNA.

The ESAC statement on the rLLNA, dated April 27, 2007 (**Annex I**), states that:

> "… the peer reviewed and published information is of a quality and nature to support the use of the rLLNA within tiered-testing strategies to reliably distinguish between chemicals that are skin sensitisers and non-sensitisers, and that animal use can be minimised providing:

> - The concentration used to evaluate sensitisation potential is the maximum consistent with solubility and the need to avoid local and other systemic adverse effects, and that this principle rather than strict adherence to the specific recommended absolute concentrations as in OECD TG 429 should be used.

> - Negative test results associated with testing using concentrations of less than 10% should undergo further evaluation.

> - Positive and negative (vehicle) control groups are used, as appropriate, per OECD TG 429.

> - The full LLNA should be performed when it is known that an assessment of sensitisation potency is required."

The ESAC statement also recommends "that further work should be undertaken to determine if the 10% concentration threshold referenced above is optimal."

1.2   Regulatory Rationale and Applicability of the rLLNA

Current regulatory testing requires assessment of the potential skin sensitization hazard of regulated substances/products. The rLLNA is being considered for use in identifying skin sensitizers in a weight-of-evidence strategy such as that proposed in the United Nations Globally Harmonised System of Classification and Labelling of Chemicals (U.N. 2005). Unlike the traditional LLNA, the rLLNA evaluates the ability of a substance to be a sensitizer based on testing a single, highest-testable dose level; therefore, dose-response information is not generated. Thus, the rLLNA is being proposed for "yes/no" identification of sensitization hazards.

1.3   Scientific Basis for the rLLNA

1.3.1   Purpose and Mechanistic Basis

The purpose of the rLLNA is to identify potential skin sensitizers by quantifying lymphocyte proliferation in the draining auricular lymph nodes after application of a test substance to the ears of a mouse. The mechanistic basis is identical to that of the traditional LLNA (see **Section 1.1.2**).

1.3.2   Applicability Domain

The applicability domain of the rLLNA should be identical to that of the traditional LLNA. The traditional LLNA was not recommended for the testing of metals, mixtures/extracts, pharmaceuticals, or strong dermal irritants (ICCVAM 1999).

1.4   Test Method Validation

The ICCVAM Authorization Act of 2000 (Sec. 4(c)) mandates that "[e]ach Federal Agency … shall ensure that any new or revised … test method … is determined to be valid for its proposed use prior to requiring, recommending, or encouraging [its use]" (Public Law 106-545, 42 United States Code 285*l*-3).

Validation is the process by which the reliability and relevance of an assay for a specific purpose are established (ICCVAM 1997). *Relevance* is the extent to which an assay will correctly predict or measure the biological effect of interest (ICCVAM 1997). For the rLLNA, relevance is determined by how well the assay identifies (1) substances capable of producing skin sensitization in humans and (2) substances that should be assessed using a diverse set of substances that represent both of the types of chemical and product classes to be tested and the range of responses to be identified.

Reliability is the reproducibility of a test method within and among laboratories. The validation process provides data and information that allow U.S. Federal agencies to develop guidance on the use of test methods in evaluating the skin sensitization potential of substances.

The first stage in this evaluation is the preparation of a draft background review document (BRD) that comprehensively reviews the relevant data and information about a test method, including its mechanistic basis, proposed uses, reliability, and performance characteristics (ICCVAM 1997). The draft BRD is made available to the public and an independent scientific peer review panel (Panel) for review and comment. ICCVAM considers these comments and those of SACATM as they finalize the BRD. ICCVAM provides the final BRD to regulatory agencies for consideration as part of the ICCVAM Test Method Evaluation Report.

1.5   Selection of Citations for the rLLNA BRD

The test method data summarized in this BRD were obtained from the original LLNA evaluation (ICCVAM 1999), peer-reviewed scientific literature, the 2007 SOT Annual Meeting, and responses to a *Federal Register* (*FR*) notice requesting such data (72 FR 27815, May 17, 2007[39]). The terms "reduced LLNA," "cut-down LLNA," "limit dose LLNA," and "limit test LLNA" were used to search MEDLINE®, TOXLINE®, and Web of Science® for publications relevant to the rLLNA test method. A review of these databases through December 2007 revealed two published reports (Kimber et al. 2006; Ryan et al. 2008 [published online ahead of print as Ryan et al. 2007]). The rLLNA was also represented at the 2007 SOT Annual Meeting in two posters (Basketter et al. 2007; Chaney et al. 2007, subsequently published as Ryan et al. 2008) and one platform presentation (Basketter 2007).

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[39]Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf