June 15, 2007

Dr William S Stokes
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Re: **72 FR 27815; May 17, 2007; National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); the Murine Local Lymph Node Assay (LLNA): Request for Comments, Nominations of Scientific Experts, and Submission of Data**

Dear Dr. Stokes:

These comments are submitted on behalf of the Alternatives Research and Development Foundation, the American Anti-Vivisection Society, Humane Society Legislative Fund, The Humane Society of the United States, People for the Ethical Treatment of Animals and the Physicians Committee for Responsible Medicine. The parties to this submission are national animal protection, health, and scientific advocacy organizations with a combined constituency of more than 10 million Americans who share the common goal of promoting reliable and relevant regulatory testing methods and strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

In January, 2007, (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the ‘‘cut-down’’ or ‘‘limit dose’’ LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated).

ICCVAM reviewed the nomination, assigned it a high priority, and proposed that NICEATM and ICCVAM carry out the following activities in its evaluation: (1) Initiate a review of the current literature and available data, including the preparation of a comprehensive background review document, and (2) convene a peer review panel to review the various proposed LLNA uses and procedures for which sufficient data and information are available to adequately assess their validation status. ICCVAM also recommends development of performance standards for the LLNA. At this time, NICEATM requests: (1) Public comments on the appropriateness and relative priority of these activities, (2) nominations of expert scientists to consider as members of a possible peer review panel, and (3) submission of data for the LLNA and/or modified versions of the LLNA.

At the meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) on June 12, 2007, several comments were made that suggested ICCVAM was assuming a relatively rapid review of these issues. However, this is not borne out by the CSPC

nomination which does not mention an expedited process. In addition, ICCVAM has recommended the creation of a background review document (BRD) and review by an expert peer review panel, with no mention of an expedited process. The cost/benefit of this LLNA review has not been evaluated, and SACATM was asked to vote to accept or reject NICEATM/ICCVAM's decision to proceed without offering any alternatives. Doubts about the cost/benefit of this project caused one SACATM member to vote against proceeding.

Despite the fact that ICCVAM documents, including the Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods,[1] mention the possibility of an expedited review process, it would appear that this process has only been used in one case. Despite repeated critiques of ICCVAM for failing to act expeditiously, we are still unable to locate a description of the expedited review process in ICCVAM literature and the parameters for applying it.

In light of the fact that the LLNA has been used by regulatory agencies for classifying skin sensitizers for years and both research data and regulatory use of the LLNA have been extensively reviewed in the literature, yet another review of this widely accepted method is unwarranted. The only circumstance under which this proposal is acceptable is if ICCVAM quickly reviews the existing literature and makes an expedited evaluation regarding the relevance of this information to Agency regulatory needs. ICCVAM's limited resources should be spent validating and promoting for regulatory acceptance any of the number of non-animal methods for skin sensitization that are currently in development.

In March 1999, ICCVAM published a final peer review report concluding that the LLNA is a valid alternative to currently accepted guinea pig test methods.[2] The U.S. EPA, FDA, and OSHA announced their acceptance of the LLNA as an alternative to the guinea pig maximization test for assessing allergic contact dermatitis in October 1999. That same year, ESAC, the Scientific Advisory Committee of the European Centre for the Validation of alternative Methods (ECVAM), also endorsed the LLNA for regulatory use.

In September 2000, the European Centre for Ecotoxicology and Toxicity of Chemicals (ECETOC) published a comprehensive review of sensitization test methods with respect to hazard identification and labeling, (and?) to determine whether the various methods are appropriate for determining relative potency and risk assessment.[3] The conclusions from this review included: (1) the LLNA is a viable and complete alternative to traditional guinea pig test methods for the purposes of skin sensitization hazard identification, and (2) the LLNA is suitable for the determination of relative skin sensitizing potency and the adaptation of this method for derivation of comparative criteria such as EC3 values provides an effective and quantitative basis for such measurements. This report further recommends that "the LLNA is the recommended method for new assessments of relative potency and/or for the investigation of the influence of vehicle or formulation on skin sensitizing potency."

[1] http://iccvam.niehs.nih.gov/SuppDocs/SubGuidelines/SD_subg034508.pdf
[2] http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm
[3] ECETOC. 2000. Skin Sensitization Testing for the Purpose of Hazard Identification and Risk Assessment.

More recent work has further verified the use of the LLNA as a stand-alone method for estimating potency for regulatory purposes, including a 2005 study that concludes that there is a "clear linear relationship between LLNA-derived EC3 values and historical human skin patch data."[4] A 2007 review concludes that "The LLNA, when conducted according to published guidelines, provides a robust method for skin sensitization testing that not only provides reliable hazard identification in formation but also data necessary for effective risk assessment and risk management." In addition, a retrospective analysis of the regulatory use of the LLNA in the EU was published in 2006 and concluded that "the LLNA is satisfactory for routine regulatory use." [5] We acknowledge that the LLNA must be validated for determining sensitization potency for regulatory use; however, we urge ICCVAM to take an abbreviated test validation approach, as was recommended by the recent International Programme on Chemical Safety Workshop on Skin Sensitization in Chemical Risk Assessment:[6] "An abbreviated test validation approach may be appropriate to assess the validity of potency assessment based on the LLNA and its appropriateness for predicting sensitizing induction potency in humans."

The ''cut-down'' or ''limit dose'' LLNA approach (reduced, or rLLNA) has recently been reviewed by an ECVAM peer review panel. In April, 2007, ESAC issued a statement supporting the use of the rLLNA "within tiered-testing strategies to reliably distinguish between chemicals that are skin sensitizers and non-sensitizers "thereby reducing animal use by as much as 50%."[7] The statement also notes the following limitations: that "the test results provided by the rLLNA do not allow the determination of the potency of a sensitising chemical," and that "negative test results associated with testing using concentrations of less than 10% should undergo further evaluation"

The applicability and limitations of this modification of the LLNA have been clearly established. Therefore, in lieu of a lengthy review of this method, ICCVAM should expeditiously review and endorse the ESAC peer review and circulate harmonized testing recommendations regarding this assay to US agencies before year's-end and NICEATM should collaborate with ECVAM to address the question of concentration threshold.

Other recent work has included the development of several applications of non-radioactive detection methods for the LLNA, including BrdU incorporation, methods measuring the release of various cytokines, and methods using fluorescent markers and quantification by flow cytometry. In many cases, these methods have been shown to be as sensitive as protocols involving radio-labeling.[8] In addition, in NIH-sponsored and contract work, MB Research has shown that "for a large range of chemicals, the FC-LLNA EC3 values were consistent with

[4] Basketter et al. Predictive identification of human skin sensitization thresholds. Contact Dermatitis. 2005; 53 (5): 260-267.
[5] Cockshott et al., The local lymph node assay in practice: a current regulatory perspective. Hum Exp Toxicol 2006; 25 (7): 387-394.
[6] http://www.who.int/ipcs/methods/harmonization/areas/sensitization_summary.pdf
[7] http://ecvam.jrc.it/publication/ESAC26_statement_rLLNA_20070525-1.pdf
[8] Takeyoshi et al. Advantage of using CBA/N strain mice in a non-radioisotopic modification of the local lymph node assay. J Appl Toxicol. 2006. 26:5-9. Takeyoshi et al. Novel approach for classifying chemicals according to skin sensitizing potency by non-radioisotopic modification of the local lymph node assay. J Appl Toxicol. 2005. 25:120-134. Suda et al. Local lymph node assay with non-radioisotope alternative endpoints. J Toxicol Sci. 2002. 27:205-218.

those reported in ICCVAM LLNA validation studies."[9] Both ECVAM and Japanese Center for the Validation of Alternative Methods (JaCVAM) are currently reviewing these methods and, rather than initiate a full independent review, ICCVAM must collaborate with these ongoing efforts.

With regard to the assessment of the LLNA for aqueous mixtures and metals, the information that is currently available should allow ICCVAM to make a rapid determination of the applicability and limitations of the LLNA for these classes of chemicals and, if it cannot, we do not endorse further validation efforts in this regard, but recommend the pursuit of *in vitro* methods for this purpose.

Several non-animal methods for estimating sensitivity are under development, including quantitative structure activity relationship (QSAR) modeling that shows a high concordance with both guinea pig and LLNA data,[10] quantification of peptide reactivity, which also shows a high concordance with LLNA data,[11] and human cell cultures.[12] We urge ICCVAM to secure an interagency grant from the CPSC to fund the validation of one or more of these non-animal methods. Clearly, ICCVAM and the CPSC both benefit from the sharing of resources, as the CPSC nominated the method and ICCVAM will be tasked with the final work product.

ICCVAM should consider taking an approach similar to the European Sens-it-iv project,[13] which involves the coordinated efforts of more than two dozen groups from industry, academia and other organizations, all working toward the common goal of developing *in vitro* methods to assess immunotoxicity. ICCVAM should consider facilitating the creation of such a goal-oriented task force.

To summarize, given the fact that the LLNA has been used by regulatory agencies for classifying skin sensitizers for years and both research data and regulatory use of the LLNA have been extensively reviewed in the literature and by other countries, yet another lengthy review of this widely accepted method is clearly unwarranted. Instead, we urge ICCVAM to perform an expedited review of the existing information regarding the LLNA's performance and limitations and to issue recommendations to US agencies with all due speed. In the interest of eventual replacement of animals in sensitization testing, ICCVAM must spend its time and resources promoting the development and regulatory use of non-animal methods, which it can do by engaging in integrated approaches to *in vitro* immunotoxicity.

Sincerely,

[9] http://www.mbresearch.com/TOXNOTE/TOXNOTE-LLNA.pdf
[10] Fedorowicz et al., Structure-activity models for contact sensitization. Chem Res Toxicol. 2005; 18(6): 954-969.
[11] Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. 2007; 97(2): 417-427.
[12] Schoeters et al. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced shin sensitization. 2007; 44(12): 3222-3233.
[13] http://www.sens-it-iv.eu/

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