

**Subject:** 74 FR 8974; February 27, 2009  
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**From:** Kate Willett  
**To:** NIEHS NICEATM <niceatm@niehs.nih.gov>

April 14, 2009

Dr William S Stokes  
Executive Director, ICCVAM  
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National Institute of Environmental Health Sciences  
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**Re: 74 FR 8974; February 27, 2009; National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of the second meeting of the Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments**

Dear Dr. Stokes:

These comments are submitted on behalf of Physicians Committee for Responsible Medicine, People for the Ethical Treatment of Animals, the Humane Society of the United States, the Alternatives Research & Development Foundation, the American Anti-Vivisection Society, and the Doris Day Animal League. These organizations represent more than ten million Americans who share the common goal of promoting regulatory testing 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) for determining potency for hazard classification; (2) the “reduced” 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 applicability domain of the LLNA.

More than a year later, ICCVAM’s Peer Review Panel reviewed findings on these five items and concluded that insufficient data existed to make recommendations about non-radioactive LLNA methods or the use of the LLNA to test mixtures,

aqueous solutions and metals. The second review panel meeting scheduled for April, 2009, is intended to reevaluate these issues in light of more recent and more complete data.

The draft recommendations resulting from this second review of the LLNA have the potential to lead to reduction or refinement of animal use in sensitization in some sectors, particularly for pesticide formulations and increased use of non-radioactive detection methods. However, we are still concerned that the time and resources that ICCVAM has devoted to this exercise has detracted from serious focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

### **Proposed applicability domain of the LLNA - mixtures, metals, and aqueous solutions**

The limited availability of data or the lack of clear definition of the test substance prevented a conclusive recommendation from the previous ICCVAM review for the use of the LLNA. Draft recommendations from the current review of formulation and aqueous solutions offer a potential for expanded use, if over-classification is accepted (presumably by both the manufacturer and the regulatory Agency). In the interim, little has changed in the availability of comparative human data and we support the review's observation that there is a need to identify relevant human data and human experience in order to continue to evaluate the applicability of LLNA to mixtures and aqueous solutions. As this approach would provide the most valuable information and does not involve further animal testing, it should certainly be a priority at this time.

During this second review, ICCVAM has come to essentially the same conclusion regarding the usefulness of the LLNA for testing metals that it had in May 2008 – that the LLNA may be useful except in the case of nickel-containing compounds.

### **Validation status of three modified (non-radioactive) LLNA test methods**

Three new methods of measuring lymphocyte proliferation have been proposed. Unlike the traditional LLNA, these new methods do not use a radioactive indicator, which could increase the use of the LLNA in facilities that cannot use radioactive material. The new protocols include two methods for detecting bromodioxymurine incorporation [BrdU-ELISA and BrdU-Flow Cytometry (FC)] and a method for detecting ATP content (LLNA: DA).

When compared to human data, the **LLNA: BrdU-FC** had a higher accuracy rate, higher sensitivity, the same specificity, the same false positive rate, and a lower false negative rate than the traditional LLNA. In order to better understand this lack of concordance, the 2008 panel requested original records for all of the studies included in the evaluation. Despite not receiving those original records, ICCVAM proceeded with the re-evaluation of this test method and, not surprisingly, arrived at a similar conclusion; that the method may prove useful; however, recommendations for use are deferred pending release of the requested data. Not only does this represent wasted effort on the part of ICCVAM and the PRP, it continues to beg the larger question of whether it is relevant to be comparing a new method, such as the LLNA: BrdU-FC, to the traditional LLNA rather than to the endpoint of actual interest, human sensitivity.

ICCVAM has concluded that it is now appropriate to recommend the **LLNA: BrdU-ELISA** and **LLNA: DA** methods with specific limitations in the decision criteria. Substances falling within an intermediate stimulation index (SI) specified for each method would be subjected to 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).” While we support this finding in general, we believe that it should be made clear that “other testing data” refers to retrospective analyses rather than initiation of additional tests in animals.

The panel also recommends that all three of these alternative detection methods be evaluated for their ability to assess mixtures, metals, and aqueous solutions concurrently with the assessment of these substances in the traditional LLNA. Since the only difference between these methods and the traditional LLNA is the method of detection, it is unlikely that there will be any differences in the applicability of these methods and the traditional LLNA with regard to mixtures, metals and aqueous solutions. Therefore, it would be highly inappropriate to perform these redundant studies, especially since there are no available data for comparison.

## **Conclusions and Future Directions**

If, based on the Draft Recommendations from this second review, the LLNA becomes a standard for pesticides formulations and if recommendations for the non-radioactive methods allow more laboratories to perform the LLNA over the Guinea Pig Maximization test or the Buehler Test, in a best-case scenario, this will

result in a moderate reduction in animal use. ICCVAM has devoted a significant portion of its resources over the past two years to these activities and we feel this is a misappropriation of ICCVAM's limited resources and do not endorse further validation efforts in this regard. Instead, we recommend that ICCVAM's limited resources be directed toward 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 guinea pig and LLNA data [1], quantification of peptide reactivity, which also shows a high concordance with LLNA data [2, 3], *in vitro* skin models [4], and human cell cultures [5, 6]. We urge ICCVAM to secure an interagency grant from the CPSC to fund the validation of one or more of these non-animal methods.

ICCVAM should consider taking a more pro-active approach similar to the European Sens-it-iv project [7], 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.

Sincerely,

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Sara Amundson  
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Doris Day Animal League

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[2] Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. *Toxicol. Sci.* 2007; 97(2): 417-427.

[3] Natsch and Emter. Skin sensitizers induce antioxidant response element dependent genes: Application to the in vitro testing of the sensitization potential of chemicals. *Tox Sci.* 2008; 102(1): 110-119.

[4] Hayden et al. 2003. *In vitro* skin equivalent modes for toxicity testing. Published in Alternative Toxicological Methods. Editors H. Salem, S.A. Katz. CRC Press LLC, Boca Raton, FL, USA, 229-247.

[5] Sakaguchi, et al., Development of an in vitro skin sensitization test using human cell lines; huna Cell Line Activation Test (h-CLAT) II. An inter-laboratory study of the h-CLAT. *Toxicol. In vitro.* 2005; 20 (5): 774-784.

[6] Schoeters et al. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced skin sensitization. *Mol. Immunol.* 2007; 44(12): 3222-3233.

[7] <http://www.sens-it-iv.eu/>