February 22, 2008

Dr William S Stokes
Director, NICEATM
National Institute of Environmental Health Sciences
PO Box 12233, MD EC-17
Research Triangle Park, NC 27709

Re: **73 FR 25553; January 8, 2008; National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of an 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 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 two 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.

Please take note of the following thoughts and transmit them to the Peer Review Panel (PRP) accordingly.

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).

Now more than a year later, ICCVAM is preparing for a peer review meeting to evaluate its recommendations and findings on these four items. It is unclear when final recommendations will be transmitted to federal agencies, but if ICCVAM's review of *in vitro* pyrogenicity methods is any indication, it may be at least another year.

Since this review of the LLNA and the proposed recommendations contained therein will lead to little reduction or refinement of animal use in sensitization, the resources that ICCVAM devote to this exercise should be kept to a minimum, and any forthcoming recommendations should be transmitted to agencies immediately following the Peer Review.

We have divided our comments into sections following the FR Notice:

LLNA limit dose procedures (the reduced or rLLNA) —draft Background Review Document (BRD) and other related documents

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%."[1]

In spite of the ESAC recommendations, ICCVAM has conducted its own data call in and data review. The reviewed database is comprehensive and contains a broad cross-section of the chemical universe. The performance characteristics were all above 95% (false negative and positive rates are very low or zero). Even though this additional review was largely unnecessary, we are pleased that ICCVAM's draft recommendations concluded favorably for the rLLNA procedure and urge the Peer Review Panel to concur. ICCVAM should forward recommendations regarding the use of the rLLNA to federal agencies immediately following the Peer Review.

Mixtures, metals, and aqueous solutions—draft Updated Assessment of the Validity of the LLNA for Mixtures, Metals, and Aqueous Solutions and related documents

ICCVAM has evaluated available data with respect to the use of LLNA in predicting the skin sensitization potential of mixtures, metals, and aqueous solutions. In all cases, the limited availability of data prevented a conclusive recommendation for the use of the LLNA; for metals, the LLNA is recommended only as part of a weight-of-evidence approach, which does not significantly promote a reduction in the use of animals.

Clearly this approach to expanding the applicability domain of the LLNA has not proved terribly fruitful, and we do not endorse further validation efforts in this regard, but recommend all resources are directed towards the pursuit of *in vitro* methods for this purpose.

Potency—draft BRD and related documents

Once again, ICCVAM has reviewed all availed data and come to a conclusion that is in opposition to that of other experts in the field. For more than 10 years data has been accumulating indicating the potential for the LLNA to make a determination of the sensitization potency of a chemical.[2] Several publications by Basketter and others (many of which are referenced in the BRD) as well as the eloquent argument by Basketter et al. presented in Appendix A, conclude that LLNA is appropriate for determining potency. 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, 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

[1] http://ecvam.jrc.it/publication/ESAC26_statement_rLLNA_20070525-1.pdf

[2] Kimber I, Basketter D A. Contact sensitization: A new approach to risk assessment. Human and Ecological Risk Assessment 1997: 3: 385 - 395.

[3] ECETOC. 2000. Skin Sensitization Testing for the Purpose of Hazard Identification and Risk Assessment.

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."

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]

Despite all of this, ICCVAM's review of the LLNA for potency determination does not support such a finding, even though, according to the BRD, the LLNA was better overall at predicting sensitization potency than guinea pig data. It is clear from the BRD that different data treatments result in different R^2 values, and the BRD should more clearly discuss the reasons those analysis decisions were made. Further, the BRD should explain in detail why conclusions were drawn that are opposite to that of the evidence they reference.

We urge the PRP to take into account the submission in Appendix A of the draft LLNA-potency BRD, which details why the LLNA is a scientifically appropriate method of potency determination, and the subsequent submitted comment by Dr. David Basketter, a recognized expert in the field of skin sensitization, when making its final report to ICCVAM.

Non-radioactive methods—draft BRDs and related documents

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 methods include two variants of a bromodioxyuridine system [BrdU: ELISA and BrdU: Flow Cytometry (FC)] and the 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. Despite this performance, the assay does not achieve complete concordance with the proposed LLNA Performance Standards the PRP will be evaluating. This is also the case with for the LLNA-DA method, which compares identically to human data, yet

[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.

falls short when compared to the traditional LLNA. While reasons for this are not clear, it is worth an examination of whether we should compare new methods to the methods they are replacing or to the endpoint of actual interest.

The BrdU: ELISA has been recommended for use by ICCVAM pending receipt of additional information and using alternative decision criteria. We support this finding. Because of the incomplete concordance between these methods and the traditional LLNA, ICCVAM qualified their acceptance and recommends a "weight-of-evidence" approach. While it is usually good scientific practice to evaluate any test method results in weight-of-evidence manner, qualifications such as these undercut the recommendations and introduce undue confusion to the reader. In our view, this gives a company a clear incentive to conduct more testing, when in reality the methods evaluated have acceptable performance and should simply be recommended.

Performance Characteristics

Although we fully support the development of performance standards that expedite the validation of new protocols that are similar to previously validated methods, we reiterate our disappointment that ICCVAM/ NICETAM has chosen to apply its limited resources to the lengthy process of developing performance standards for such a narrow scope of applicability. These performance standards apply only to modifications of the "standard LLNA" that involve incorporation of non-radioactive methods of detecting lymphocyte proliferation.

In addition, the draft performance standards require the use of a minimum of 22 reference compounds. The criteria by which the compounds were chosen and the characteristics of the compounds are described; however, there is no justification for the requirement of such a large number of compounds for this particular method modification. The methods to which these performance standards apply will differ from the "standard LLNA" only in the method of detection of lymphocyte proliferation; therefore the element of concern is sensitivity of the detection method. All other aspects of the methods to be evaluated will be identical to the standard LLNA, including delivery and biological response. It is therefore not necessary to test representatives for every chemical class or every solvent that has been tested in the standard LLNA. The important characteristic of the reference compound is the magnitude of proliferation response that is generated, and the list of reference compounds chosen should be limited to those that represent the range of response seen with the standard LLNA.

In addition, a major criterion for the selection of the above compounds is that there are Guinea pig data available; more appropriately, chemicals should be chosen on the basis of available human data.

Conclusions and Future directions

This exercise is a good example of actions undertaken by ICCVAM which result in frustration in the animal protection community. In the future we hope that ICCVAM will take a more holistic approach to determine the ways in which it spends its limited time and resources so as to ensure maximum benefit for animals in laboratories.

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,[6] quantification of peptide reactivity, which also shows a high concordance with LLNA data,[7,8] and human cell cultures.[9,10] 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 a more pro-active approach similar to the European Sens-it-iv project,[11] 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,

/s/

Catherine Willett, PhD
Science Policy Advisor
Regulatory Testing Division
People for the Ethical Treatment of Animals

/s/

Kristie Stoick, MPH
Scientific and Policy Advisor
Physicians Committee for Responsible Medicine

[6] Fedorowicz et al. Structure-activity models for contact sensitization. Chem Res Toxicol. 2005; 18(6): 954-969.
[7] Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Toxicol. Sci. 2007; 97(2): 417-427.
[8] 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.
[9] 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.
[10] Schoeters et al. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced shin sensitization. Mol. Immunol. 2007; 44(12): 3222-3233.
[11] http://www.sens-it-iv.eu/