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Experience and Innovation

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Dr. Mary Wolfe
Director, NTP Liaison and Scientific Review Office
NIEHS/NIH
P.O. Box 12233, MD A3-01
111 TW Alexander Drive
Research Triangle Park, NC 27709

RE: Nominations to ICCVAM, Non-Radioactive Murine Local Lymph Node Assays, Request for Comment, *Federal Register, Vol. 72, No.83, pages 23831-23832, May 17, 2007*

Dear Dr. Wolfe and Honorable Committee Members:

In response to the Consumer Product Safety Commission's request to NICEATM and ICCVAM to evaluate non-radioactive versions of the Local Lymph Node Assay (LLNA), MB Research Laboratories would like to offer its support for this nomination and extend our assistance and available information towards the validation of non-radioactive LLNA methods.

MB Research Laboratories has developed and routinely performs a commercial research protocol for the assessment of acute dermal sensitization using a **Flow Cytometry-based Local Lymph Node Assay – FC-LLNA**. In contrast to the radioactive LLNA, the FC-LLNA assesses proliferation by determining incorporation of the thymidine analog bromodeoxyuridine (BrdU) into the DNA of lymph node cells, along with evaluation of lymph node cell number, using flow cytometric methods. It is safer to conduct because of the elimination of hazardous radioactive material, and with added endpoints, is able to better identify true sensitizers and false positive irritants.

The FC-LLNA is a direct result of a three-year SBIR grant project (R44-ES-10234-02). The goal of the project was to develop a commercially viable assay that would be a significant improvement over the standard radioactive LLNA while maintaining high levels of accuracy, sensitivity, specificity, and predictivity. During the conduct of our internal validation studies, over 50 chemicals, including sensitizers, nonsensitizers and irritants were tested. Since 2001, more than 80 FC-LLNA studies have been conducted by clients in the chemical, pharmaceutical, and consumer product industries for safety evaluations and potential submission to regulatory agencies.

The FC-LLNA is very similar to the ICCVAM-validated LLNA protocol but adapted for flow cytometric evaluation. Specifically, the dosing method, assay schedule, vehicles and positive controls are identical. Of the similarities, most noteablely both assays evaluate lymphocyte proliferation and designate a cut off value of stimulation index (SI) = 3 as a positive indication of sensitization.

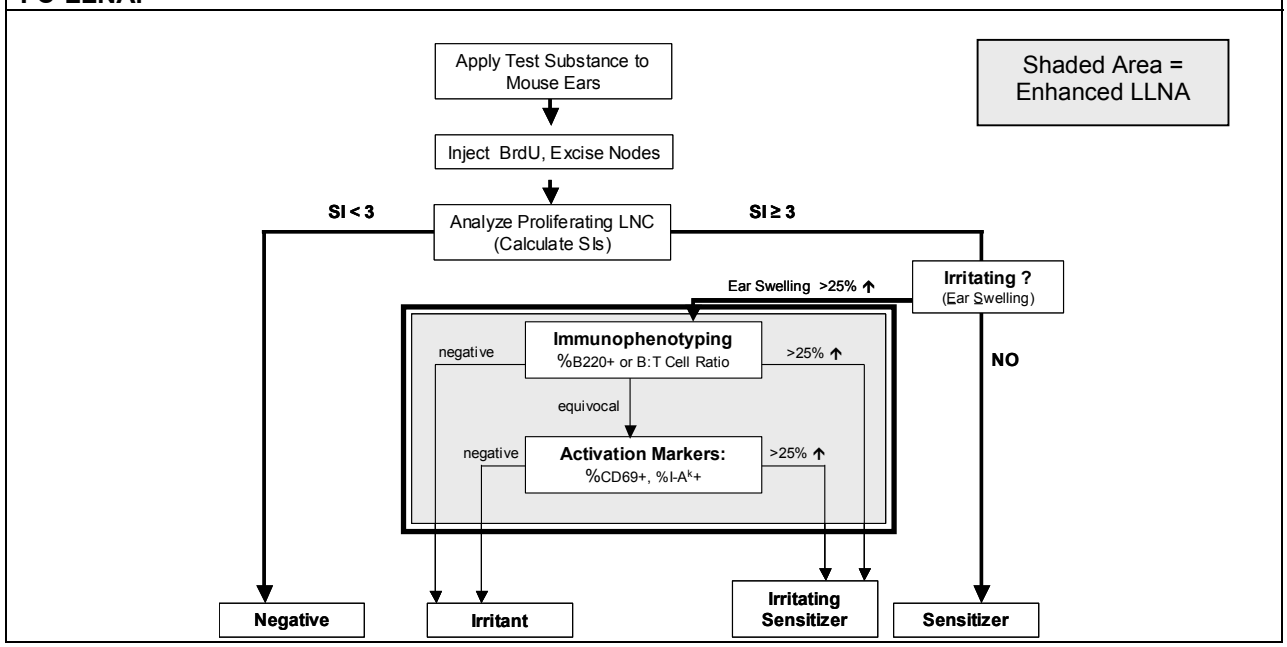
The significant difference between the two protocols is that in the radioactive LLNA mice are injected by tail vein with ^{3}H thymide, while in the FC-LLNA mice are injected intraperitonially with BrdU. Additionally, because the cells are not radiaoactively labeled, an aliquot can also be stained for immunophenotyping and activation marker analysis, thus reducing the need for additional animal groups. Profiling of immunophenotypic markers such as B220, CD3, I-A^{k} and CD69 can be added to our basic protocol to distinguish between sensitizers and false positive irritants. Ear swelling measurements have also been included to the basic FC-LLNA test to evaluate irritation of test articles and screen for possible false positives.

In the FC-LLNA, proliferation of lymph node cells is measured by a combination of BrdU incorporation and total lymph node cell number. As with the radioactive version of the LLNA, an SI of 3 or greater indicates a positive sensitizing response. Each treatment group consists of five mice. Each mouse is evaluated independently by multiplying the total number of lymphocytes by the percentage of lymph node cells that are positive for BrdU incorporation. The total number of proliferating cells in the test group is divided by the total number of proliferating cells in the vehicle group to give a stimulation index. The FC-LLNA yields SI's similar to those in the ICCVAM validation report as well as other published results for the radioactive LLNA. The estimated concentration of chemical required to induce an SI of 3 (EC3), can be used to determine the potency of sensitizers. EC3 values obtained in the cytometric LLNA are quite comparable to those found in the radioactive LLNA, and in most cases fall within the range of values obtained for chemicals tested in the radioactive assay. (See Table 1)

For our validation, immunophenotype analysis of the nodal cells was conducted using the marker combinations B220/CD3 to determine the ratio of B cells to T cells and I-A^k/CD69 to determine the activation state of the nodal lymphocytes. More specifically, to investigate activation state, the murine MHC class II alloantigen (IA) surface marker was evaluated and the percentage of the total nodal percentage of I-A^k+ cells that were also positive for the CD69 marker was determined. A major advantage of the FC-LLNA is that immunophenotype analysis can be performed on an aliquot of the cells harvested for SI analysis and no additional animals need be used.

An illustration of the FC-LLNA multi-tiered approach to evaluate sensitizers and eliminate false-positive irritants is shown in Figure 1.

Figure 1. Multi-tiered Testing Strategy for the Assessment of Sensitization Potential using the FC-LLNA.



In the first tier, an SI<3 indicates a non sensitizer. For chemicals that elicit an SI>3, ear thickness measurements can be utilized as an indication of irritancy, since CBA mice are brown, thus erythema cannot be evaluated. In the second tier of our FC-LLNA, positive ear swelling flags possible false positive irritants due to the fact that irritants dramatically increase the thickness of the ear, while contact allergens induce a minimal increase in skin thickness due to low inflammatory response. In the last tier, immunophenotyping markers are used to distinguish between true sensitizers and false positive irritants. These markers strongly correlate to positive sentization potential. Additionally, we have found that some irritants do not increase ear swelling, but can be distinguished from sensitizers because of a lack of immunophentypic response.

Table 1 is a list of compounds tested in the FC-LLNA compared to the radioactive LLNA based on SI alone. Also included in the table are a group of equivocal compounds, which were not included in contingency table evaluations.

Table 1: LLNA Compound List Comparing MB Research Flow Cytometry (FC) LLNA Results with ICCVAM Validation Radioactive (R) LLNA Results

Positive by Radioactive LLNA	FC	R
2,4-dinitrochlorobenzene	+	+
Aminophenol HCL	+	+
Benzoyl peroxide	+	+
Chlorpromazine +UVR	+	+
Citral	+	+
Cobalt chloride	+	+
Copper chloride	+	+
Croton Oil	+	+
Diethylenetriamine	+	+
Diphenylcyclopropenone	+	+*
Ethylene glycol dimethacrylate#	+	+
Eugenol	+	+
Fluorescein isothiocyanate	+	+
Formaldehyde	+	+
Hexylcinnamaldehyde	+	+
IsoEugenol	+	+
Isopropyl Myristate	+	+*
Linalool	+	+*
Oxazolone	+	+
Potassium dichromate	+	+
p-phenylenediamine	+	+
Pyridine	+	+
Sodium lauryl sulfate#	+	+
Tetrachlorosalicylanilide	+	+
Trimellitic anhydride	+	+*
Xylene	+	+

Negative by Radioactive LLNA	FC	R
6-methyl coumarin	–	–
Benzoic acid	–	–
Chlorobenzene	–	–
Glycerol	–	–
Hexane	–	–
Hydrocortisone	–	–
Isopropanol	–	–
Lactic acid	–	–
Methyl salicylate	–	–
Nickel chloride	–	–
p-aminobenzoic acid	–	–
Propylene glycol	–	–
Propylparaben	–	–
Resorcinol	+	–
Sulfanilamide	–	–
Tween 80	+	–

Equivocal	FC	R
Aniline	–	+/–
Benzalkonium chloride#	+	+/–
Benzocaine	+/–	+/–
Ethylenediamine	+	+/–
MBT	+/–	+
Salicylic acid	+/–	–

* = HSE contract research report 399, 2001. Development of the Local Lymph Node Assay for Risk Assessment of Chemicals and Formulations, Rebecca J. Dearman and Ian Kimber, Syngenta Central Toxicology Laboratory, UK, 2001, p.12.
# = Classify as irritants but not sensitizers using the enhanced FC-LLNA with immunophenotype endpoints.

We have also provided in Table 2, a comparative evaluation of data from the flow cytometric assay (FC), the radioactive assay (R), guinea pig results (GP) and human data (H). The cytometric assay has 95% accuracy to the radioactive assay, as well as 93% sensitivity and 100% specificity. Moreover, while the FC-LLNA is less accurate than the radioactive assay when compared to the guinea pig assay (79% vs. 89%) it is more accurate than the radioactive test when compared to human data (88% vs. 72%).

Table 2: Comparative Evaluation of the Flow Cytometric LLNA

Comparison of Method	Total #	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity	
		%	#	%	#	%	#	%	#	%	#
FC-LLNA vs. R-LLNA	42	**95%**	40/42	**93%**	26/28	**100%**	14/14	**100%**	26/26	**88%**	14/16
FC-LLNA vs. Human	26	88%	22/25	90%	18/20	83%	5/6	95%	18/19	71%	5/7
R-LLNA vs. Human	74	72%	53/74	72%	49/68	67%	4/6	96%	49/51	17%	4/23
FC-LLNA vs. Guinea Pig*	29	79%	23/29	74%	14/19	90%	9/10	93%	14/15	64%	9/14
R-LLNA vs. Guinea Pig*	97	89%	86/97	91%	62/68	83%	24/29	93%	62/67	80%	24/30

Radioactive LLNA results obtained from ICCVAM Validation of the LLNA[b]
* = Results from Guinea Pig Maximization Test and/or Beuhler Assay

Augmentation of the original LLNA with the flow cytometry endpoints increases the sensitivity and discriminating power of the LLNA, while (1) complying with the Animal Welfare Act by directly addressing the reduction in animal number; (2) increasing the quality and quantity of data generated when compared to existing methods; and (3) substantially reducing the cost of analysis and waste disposal by avoiding the use of radioactivity.

In conclusion, MB Research Laboratories fully supports the Consumer Product Safety Commission's nomination to ICCVAM for the evaluation of the non-radioactive LLNA methods for classifying sensitizers and offers to assist ICCVAM by offering the FC-LLNA protocol, validation data and methods for consideration as a direct substitute to the Guinea Pig Sensitization Test.

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