MB Research Labs
Experience and Innovation

TOXNOTE

Local Lymph Node Assay using Flow Cytometric Endpoints

An Alternative to the Radioactive LLNA

Problem: Irritant or Irritating Sensitizer?

The local lymph node assay (LLNA) is an alternative to the guinea pig sensitization test used to identify and characterize dermal sensitizers. MB Research has optimized procedures and applied flow cytometric techniques to the EPA and OECD LLNA protocol in order to enhance the basic LLNA and to increase sensitivity/specificity. The validation of the FC-LLNA is the result of several years of research and funding by the National Institutes of Health, in which an expanded set of chemicals (known sensitizers, sensitizing irritants and non-sensitizers) were evaluated using the FC-LLNA to determine the predicitivity and reproducibility of the assay.

Due to the need for radioactive nucleotides to measure Lymph Node Cell (LNC) proliferation, special licensing is needed to perform the standard LLNA . In comparison, the FC-LLNA utilizes BrdU incorporation into the DNA of proliferating LNCs, which is safer and drastically reduces the overall study costs. In addition, accuracy is enhanced by the ability to perform immunophenotypic and surface marker (activation) analysis on the isolated lymph node cells.

The additional quantification endpoints of the FC-LLNA have been successfully used by leaders in chemical and pharmaceutical industries to further characterize test compounds that have proven difficult to classify as irritants or irritating sensitizers using standard testing methods.

The FC-LLNA is a possible solution to the problem.

For more information, see: WWW.LLNA.COM

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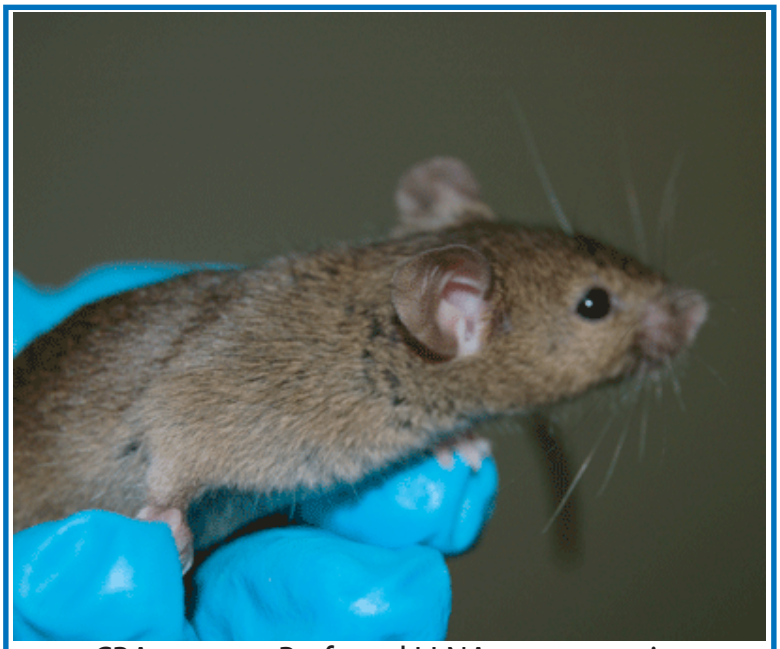
BACKGROUND:

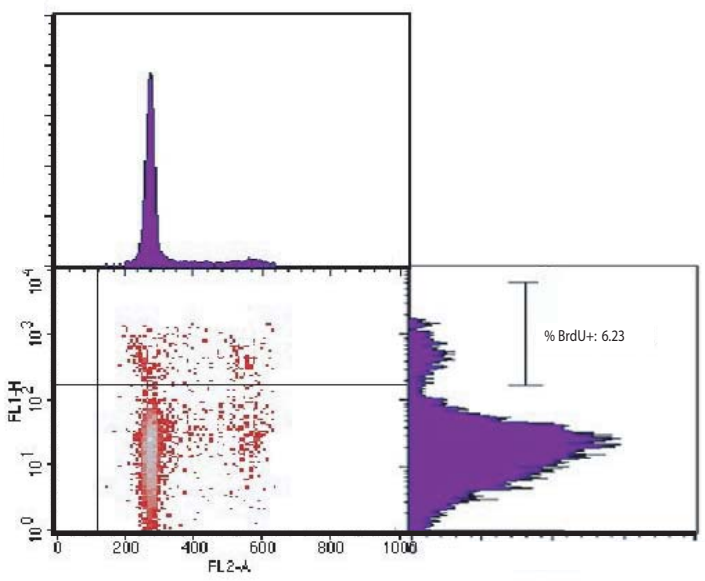
Allergic Contact Dermatitis is a common occupational health problem. The local lymph node assay is an animal-based toxicology test developed as an alternative to the transdermal guinea pig sensitization test. This guinea pig test has been long used as the gold standard assay for the EPA, FDA, OECD and industry to identify and characterize substances with immunotoxic properties. The LLNA has been accepted by ICCVAM as a stand-alone alternative to the Guinea Pig Maximization Test and the Buehler Assay. The traditional form of the LLNA employs a single radioactive endpoint to measure LNC proliferation. While this assay is effective at detecting potential irritants/sensitizers, it cannot readily differentiate some types of irritants and sensitizers.

MB Research Labs has optimized procedures and applied flow cytometric techniques to the EPA and OECD LLNA protocol in order to enhance the basic LLNA and to increase sensitivity/specificity. Proliferation is determined by measurement of total LNC count and BrdU incorporation into DNA, which eliminates the need for radioactivity. Moreover, cytometric immunophenotype analysis and quantitative ear thickness measurements have been added. These additional endpoints provide a comprehensive tiered testing strategy to provide better quantitative and qualitative information regarding the immunotoxic effects of various chemical compounds. Proliferation of cells in the lymph node is represented as Stimulation Index, which takes into account cell number and incorporation of BrdU into proliferating cells. As in the radioactive form of the LLNA, a Stimulation Index of 3 or greater indicates a positive sensitizing response.



CBA mouse - Preferred LLNA mouse strain

Proliferation of LNC - BrdU Incorporation



Proliferation is assessed by determining the incorporation of the thymidine analog bromodeoxyuridine (BrdU) into the DNA of LNCs using flow cytometric method.

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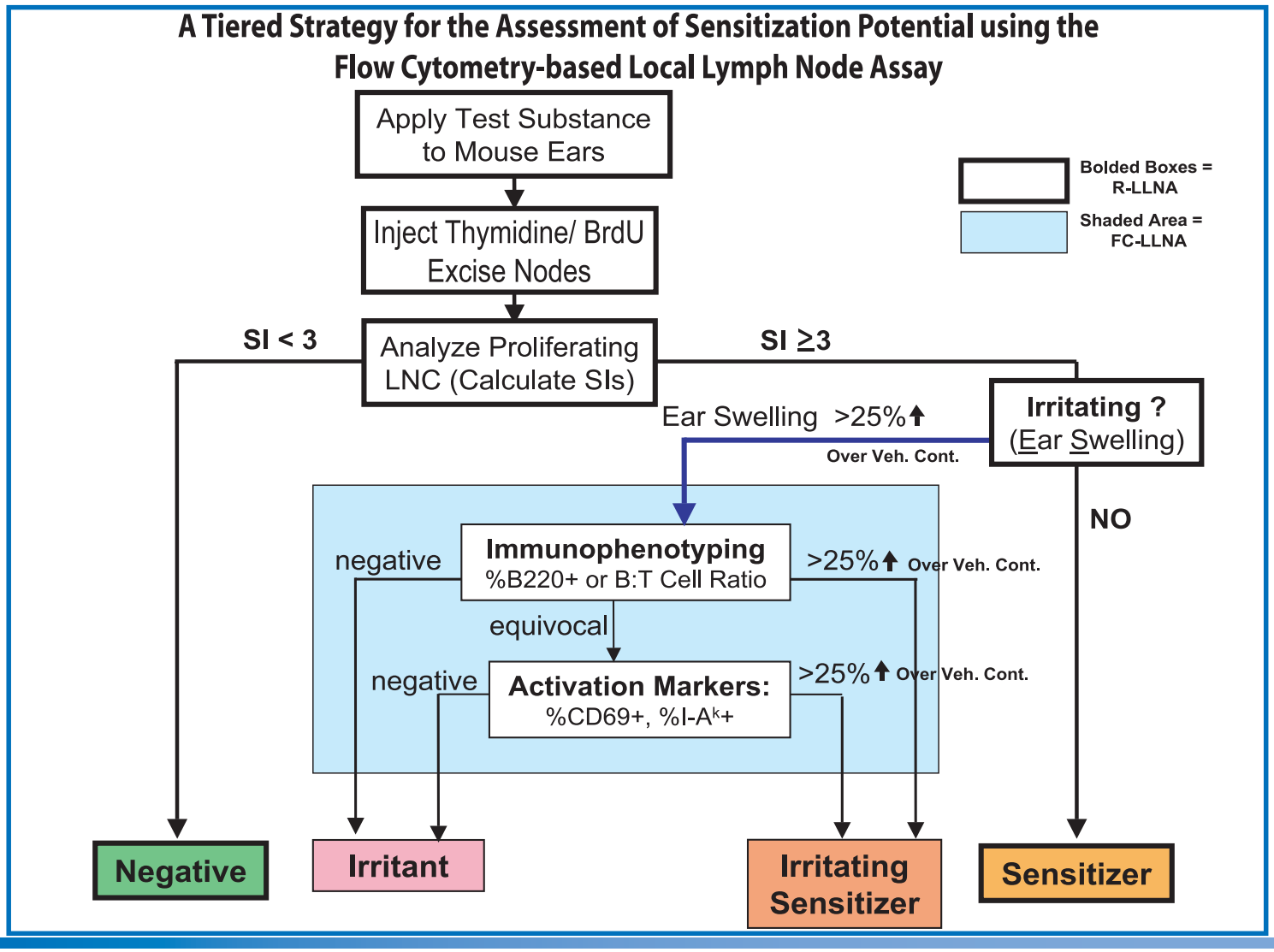
Procedural Outline:

1. Determine optimal vehicle for test article (solubility testing)
2. Conduct Dermal Irritation Pre-Screen to determine test concentrations
3. Dose ears once daily for 3 consecutive days
4. On Day 6, (2 day rest period) inject mice with BrdU
5. Sacrifice and isolate the auricular lymph nodes
6. Process/stain individual animals' lymph node cells (LNC)
8. Flow cytometric measurement of proliferation of LNC
9. Determine the Stimulation Index (S.I.) for each group
10. Calculate, if possible, the EC3 (Minimal Sensitizing Concentration)*

* Concentration at which SI = 3, the OECD and EPA criteria for positive sensitizers.



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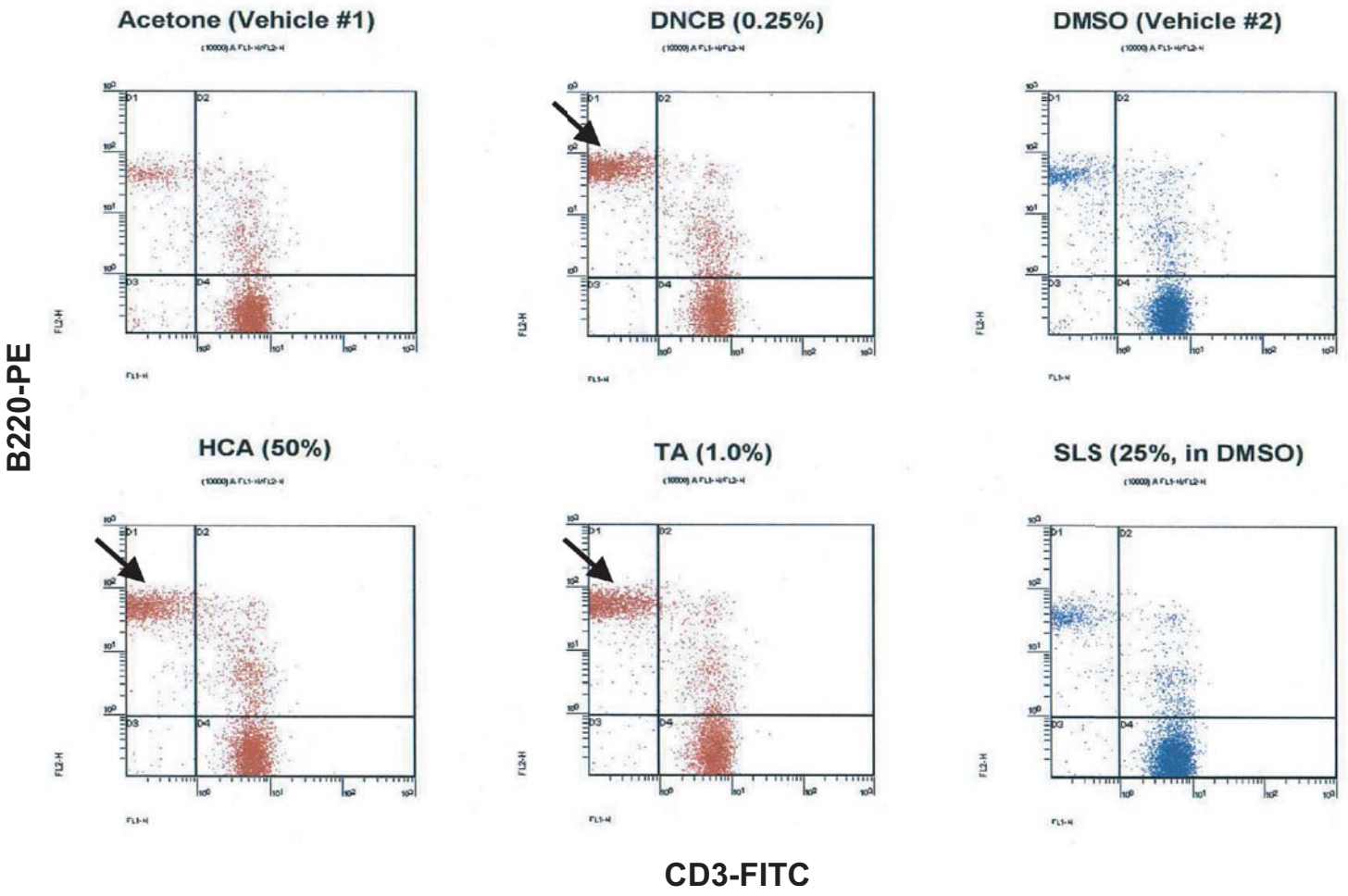
RESULTS:

Increase in B220+ Lymphocytes Correlates with Sensitizers but not False Positive Irritants



A major component of the FC-LLNA involves the routine inclusion of immunophenotype endpoints. It is generally known that some irritants such as SLS can induce false positive responses in the standard radioactive LLNA, erroneously identifying them as sensitizers. However, it is also known that these agents induce a major alteration in the types of cells present in the auricular lymph node following treatment. Therefore, immunophenotype analysis of the node would help identify these false positives (irritants) and increase sensitivity and specificity of the assay.

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RESULTS:

Table 2: Dermal Sensitization Comparison by Test Method

Positive	FC	R	G	H
2,4-dinitrochlorobenzene	+	+	+	+
Aninophenol HCL	+	+	+	
Benzoyl peroxide	+	+	+	+
Chlorpromazine +UVR	+	+	+	+
Citral	+	+	+	+
Cobalt chloride	+	+	+	+
Copper chloride	+	+	–	
Croton Oil	+	+		
Diethylenetriamine	+	+	+	+
Diphenylcyclopropenane	+	+*		
Ethlene 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	FC	R	G	H
6-methyl counarin	–	–	–	–
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**	**G**	**H**
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.

FC = Flow Cytometry-based LLNA R = Radioactive LLNA G = Guinea Pig Results H = Human Results

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RESULTS:

Table 3: Comparative Evaluation of the Flow Cytometry-based 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%	51/15	64	9/14
R-LLNA vs. Guinea Pig*	97	89%	86/97	91%	62/68	83%	24/29	93%	14/67	80	24/30

Radioactive LLNA results obtained from ICCVAM Validation of the LLNA
* = GPMT/BA Results

SUMMARY

We have developed a Flow Cytometry-based Local Lymph Node Assay (FC-LLNA) that is a significant improvement on the existing radioactive LLNA (R-LLNA). In place of 3H-labeled thymidine, we measure LNC proliferation based on cell number and incorporation of BrdU. Additional endpoints of the FC-LLNA definitively resolve false positive irritants from true dermal sensitizers (Delayed Type IV Hypersensitivity).

Compared to the radioactive LLNA, our standard flow cytometry-based LLNA has 95% accuracy, 93% sensitivity, 100% specificity, 100% positive predicitivity and 88% negative predictivity. By using the added immunophenotyping (B cell and T cell subtypes) and immunoactivation marker (IAK and CD69) endpoints of the enhanced assay, we are able to characterize false positive irritants, including Sodium Lauryl Sulfate (SLS) and Benzalkonium Chloride (BAC), as well as some weak human sensitizers including, Tween 80 and Resorcinol.

CONCLUSIONS

- The LLNA can be conducted by flow cytometry and without radioactivity using the thymidine analog BrdU. For a large range of chemicals, the FC-LLNA EC3 values were consistent with those reported in ICCVAM LLNA validation studies.
- Ear swelling measurements assist in determining optimal or maximal dose ranges and in identifying false positive irritants, e.g. SLS.
- Treatment with known sensitizers induces increases in B cells and concurrent decrease in T cells (elevated B:T ratio) in the nodes of treated animals.
- Immunophenotypic parameters such as %B220+, %CD3+, %CD69+ and %IAk+ in nodal cells are useful endpoints

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