Final Validation of a Flow Cytometry-based Local Lymph Node Assay with Immunophenotypic Endpoints

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ABSTRACT

The Local Lymph Node Assay (LLNA) is an alternative to the guinea pig sensitization test used to identify and characterize dermal sensitizers. We have 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.

Currently, special licensing is needed to perform the LLNA, due to the need for radioactive nucleotides to measure Lymph Node Cell (LNC) proliferation. In comparison the Flow Cytometry-based 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.

Four commercial versions of the FC-LLNA have been developed and is now offered as a GLP-compliant alternative assay to the consumer products, chemical and pharmaceutical industries.

In summary, we have developed an enhanced flow cytometry-based LLNA which 1) can sensitively and specifically identify and characterize dermal contact sensitizers; 2) does not require radioactivity; 3) allows better discrimination of false-positive irritants from true sensitizers when compared to the basic radiometric LLNA, and 4) can be a more accurate and quantitative tool for assessment of potential dangerous substances to public.

INTRODUCTION AND 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 (GPMT) and the Buehler Assay (BA). The current form of the assay employs a single radioactive endpoint to measure lymph node cell proliferation. While this assay is effective at detecting potential irritants/sensitizers, it cannot readily differentiate some types of irritants and sensitizers. To enhance and improve this important screen, MB Research Laboratories has applied flow cytometric techniques to this assay. 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 has 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 absolute 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.

METHODS AND MATERIALS

The ICCVAM LLNA protocol was followed in detail, with the exceptions identified below. All test materials were applied topically once daily for 3 days to the dorsum of each ear of female CBA/J mice (8-12 weeks old; n = 5). Three days after the last treatment, the mice were injected with the thymidine analog BrdU, which then becomes incorporated into proliferating lymph node cells. Five hours after injection, the mice were euthanized and the auricular nodes were collected and pooled on an individual animal basis. Lymph nodes were processed into single-cell suspensions in a microtube with a disposable pestle in PBS. The cells were centrifuged, washed, and re-suspended in cold PBS and refrigerated overnight.

Immunophenotyping: Aliquots from each LNC suspension (~ 5 x 10^5 cells) were stained with fluorescently-conjugated B cell, T cell and cell surface marker-specific antibodies (B220, CD3, CD4, CD8, I-A^k, CD69, CD25. CD44 and CD62L) obtained from BD Pharmingen and then analyzed by flow cytometry.

Cell Proliferation (total LNC number): Aliquots of LNC were fixed by re-suspending in 70% EtOH. Fixed cells were acid and TX-100 permeablized, neutralized and stained with FITC-conjugated anti-BrdU antibody for flow cytometric analysis. The %BrdU-positive was determined and number of proliferating (BrdU+) cells was calculated. The number of BrdU+ LNC is calculated using the following equation:

Total # cells per node set X [%BrdU+] = # BrdU+ cells/mouse

The stimulation index (SI) of a compound is the ratio of the mean # of proliferating [BrdU+] lymph node cells in test article-treated groups relative to # of BrdU+ LNC in vehicle-treated groups. Test articles that yield a SI $\geq$ 3 are characterized as sensitizing substances.

$$\frac{\text{\#BrdU+ LNC from treated group}}{\text{\#BrdU+ LNC from vehicle group}} = \text{SI}$$

For each group, the mean # of BrdU+ LNC was calculated and divided by the vehicle control group to obtain the SI for each individual animal. Flow cytometry was conducted using a GLP-validated, enhanced FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an Omnichrome 25 mW argon laser emitting at 488 nm with 15 mW of power and an Automated Microsampling System (Cytek Development, Fremont, CA). The histograms generated in these experiments were analyzed using WinFCM Software (Applied Cytometry Systems, Sheffield, UK) and CellQuest Flow Cytometry Software (Becton Dickinson, San Jose, CA).

RESULTS

Figure 1

A Tiered Strategy for the Assessment of Sensitization Potential using the FC-LLNA

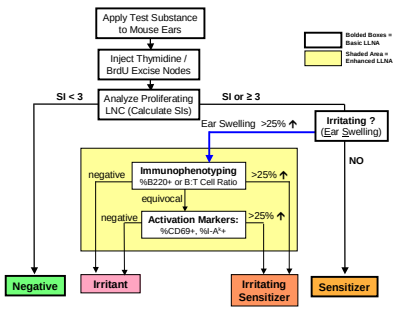

Figure2

LNC Proliferation: % BrdU+ LNC Measurement

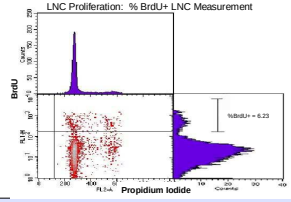

Figure 9

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

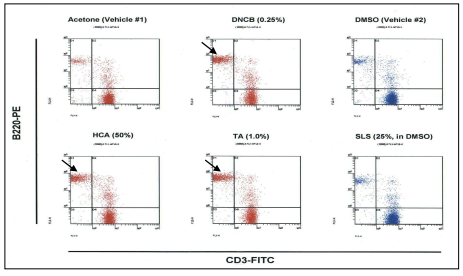

Table 1: Comparison of EC_3 Values

FC-LLNA is Quantitatively Comparable to R-LLNA

Classification	Chemicals	Human/Guinea Pig Results[a]	Historical LLNA EC_3	MBR FC-LLNA EC_3
Sensitizer	Oxazolone	Strong	0.003 –0.02%[b]	0.01 – 0.02%
	Diphenylcyclopropenone	Strong	0.05%[c]	0.03%
	2,4-DNCB	Strong	0.03 – 0.09%[b]	0.01 – 0.09%
	TCSA	Strong	0.04 – 0.17%[b]	0.06%
	p-Phenylenediamine	Strong	0.06%[c]	0.45%
	Formaldehyde	Moderate	0.39%[c]	0.29%
	HCA	Moderate	7.0 – 12.2%[c]	6.3%
	Citral	Weak	13%[c]	2.0%
	Eugenol	Weak	13%[c]	13.2%
	Linalool	Very Weak	30%[c]	31.0%
	Ethyleneglycol dimethacrylate	Very Weak	35%[c]	40.0%
	Isopropyl myristate	Very Weak	44%[c]	12.1%
	Propyl paraben	Very weak	>50%[c]	>50%
	Benzocaine	Very Weak	> 25%[c]	> 50%
Non-Sensitizer (Irritant)	Benzalkonium Chloride	Irritant	> 2.5%[c]	0.4%
	SLS	Irritant	6.9 – 17.1%[c]	4.84%
	Ethylenediamine	Irritant	4.4 – 10.7%[c]	2.7%
Non-Sensitizer (Non-Irritant)	Hexane	Non-Sensitizer	> 100%[c]	>100%
	Surfanilamide	Non-Sensitizer	> 50%[c]	>50%
	PABA	Non-Sensitizer	>10%	16.9%
	Isopropanol	Non-Sensitizer	> 50%[c]	>50%

a = 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.

b = ICCVAM REPORT: The Murine Local Lymph Node Assay, Results of an Independent Peer Evaluation Coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM), Feb 1999, p.22 and L2.

c = R. J. Dearman, Z. M. Wright, D. A. Basketter, C. A. Ryan, G. F. Gerberick, and I. Kimber. The suitability of hexyl cinnamic aldehyde as a calibrant for the murine local lymph node assay. *Contact Dermatitis* 44 (6):357-361, 2001.

Figure 2

Stimulation Index Values of Sensitizers, Irritants, and Non-Sensitizers

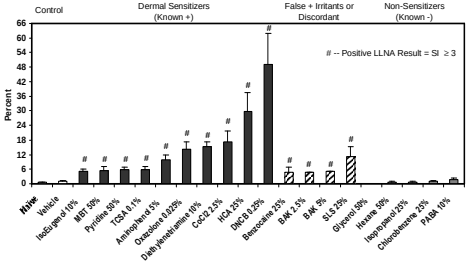

Figure 3

Ear Swelling: Percent Changes over Pre-Test Values

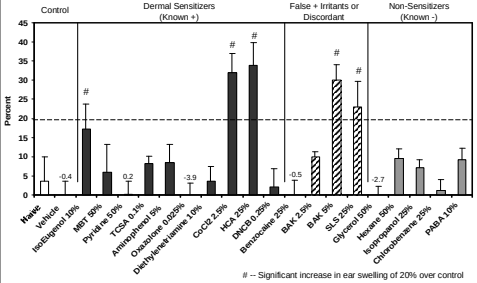

Figure 4

Percent B220+ Lymphocytes (B Cell Subset) in Lymph Nodes

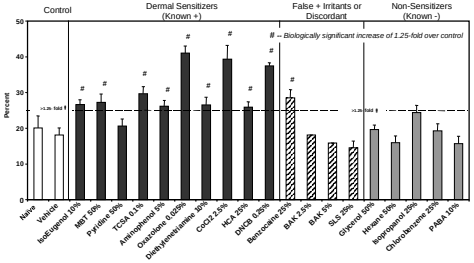

Figure 6

Percent I-A^k+ Cell Subset in Lymph Nodes

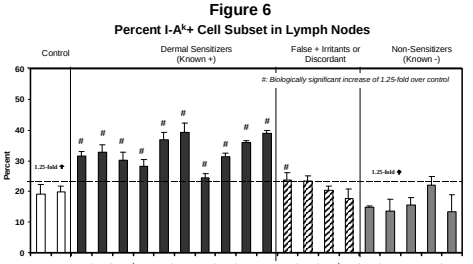

Table 2

Dermal Sensitization Comparison by Test Method

Positive	FC	R	G	H	Negative	FC	R	G	H
2,4-dinitrochlorobenzene	+	+	+	+	6-methyl coumarin	–	–	–	–
Aminophenol HCL	+	+	+		Benzoic acid	–	–		–
Benzoyl peroxide	+	+	+	+	Chlorobenzene	–	–	–	
Chlorpromazine +UVR	+	+	+	+	Glycerol	–	–	–	
Citral	+	+	+	+	Hexane	–	–		–
Cobalt chloride	+	+	+	+	Hydrocortisone	–	–		–
Copper chloride	+	+	–		Isopropanol	–	–	–	
Croton Oil	+	+			Lactic acid	–	–	–	
Diethylenetriamine	+	+	+	+	Methyl salicylate	–	–	–	–
Diphenylcyclopropenone	+	+*			Nickel chloride	–	–	+	
Ethylene glycol dimethacrylate	+	+	–	+	p-aminobenzoic acid	–	–		
Eugenol	+	+			Propylene glycol	–	–	–	
Fluorescein isothiocyanate	+	+			Propylparaben	–	–	–	+/–
Formaldehyde	+	+	+	+	Resorcinol	+	–	–	+
Hexylcinnamaldehyde	+	+	+	+	Sulfanilamide	–	–	–	+
IsoEugenol	+	+	+	+	Tween 80	+	–	–	+
Isopropyl Myristate	+	+*			**Equivocal**	**FC**	**R**	**G**	**H**
Linalool	+	+*		+	Aniline	–	+/–	+	+
Oxazolone	+	+	+	+	Benzalkonium chloride	+	+/–	–	+
Potassium dichromate	+	+	+	+	Benzocaine	+/–	+/–	+/–	+
p-phenylenediamine	+	+	+	+	Ethylenediamine	+	+/–	+	+*
Pyridine	+	+		+	MBT	+/–	+	+	+
Sodium lauryl sulfate	+	+	–	–	Salicylic acid	+/–	–	–	–
Tetrachlorosalicylaniliide	+	+	+	+					
Trimellitic anhydride	+	+*							
Xylene	+	+		–					

*=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

Table 3

Comparative Evaluation of the Flow Cytometric LLNA

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	28	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*

* = GPMT/BA Results

SUMMARY

We have developed a Flow Cytometry-based Local Lymph Node Assay (FC-LLNA) that is significantly improved on the existing radioactive LLNA (R-LLNA). In place of ^{3}H-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). Ear swelling measurements are performed in our standard LLNA.

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) immunoactivation marker (IA^k 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 EC_3 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 %IA^k+ in nodal cells are useful endpoints for discriminating false positive irritants from true sensitization responses.

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