

USE OF AN ENHANCED LOCAL LYMPH NODE ASSAY TO CORRECTLY CLASSIFY IRRITANTS AND FALSE POSITIVE SUBSTANCES.

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ABSTRACT

A major weakness of the local lymph node assay is that irritants can produce increases in the stimulation index (SI>3) in a manner analogous to true sensitizers or allergens. Due to the short-duration of the LLNA, many known human irritants are not detected as such during the course of the test (ear swelling and edema). The prototypical false positive irritant in the LLNA is SLS at concentrations above 20-25%. Herein we use an enhanced LLNA with immunophenotypic endpoints and measurements of ear thickness to characterize, identify, and correctly classify additional irritants, including benzalkonium chloride and ethylenediamine. Treatment with these irritants can result in maximum SI values from 5.4 to 13.3. Using flow cytometric analysis of surface markers on lymphocytes, including B220, CD3, I-Ak (MHC), and CD69, we are able to distinguish true sensitizers from these from other anomalous test substances that increase Stimulation Index by unknown mechanisms. None of the false positive irritants exhibited the hallmarks of true sensitizers, specifically increases in percent B220+, I-Ak + and cd69/iak++ cells. In contrast, irritants tended to decrease the percent of B-lymphocytes while increasing the percent of T-lymphocytes in the draining lymph node. Thus, in the event that irritancy is present or suspected as a known property of the test substance, we herein define a tier-testing approach which integrates additional endpoints including ear thickness measurements and immunophenotypic analysis. This Enhanced LLNA (E-LLNA) allows a more thorough assessment and accurate classification of problematic substances.

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 10⁵ 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 permeabilized, 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:

$$\text{Total \# cells per node set} \times [\% \text{BrdU+}] = \# \text{ BrdU+ cells/mouse}$$

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 ≥ 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 Omnicrome 25 mW argon laser emitting at 488 nm with 15 mW of power and an Automated Microsampling System (Cytex 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).

Figure 1

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

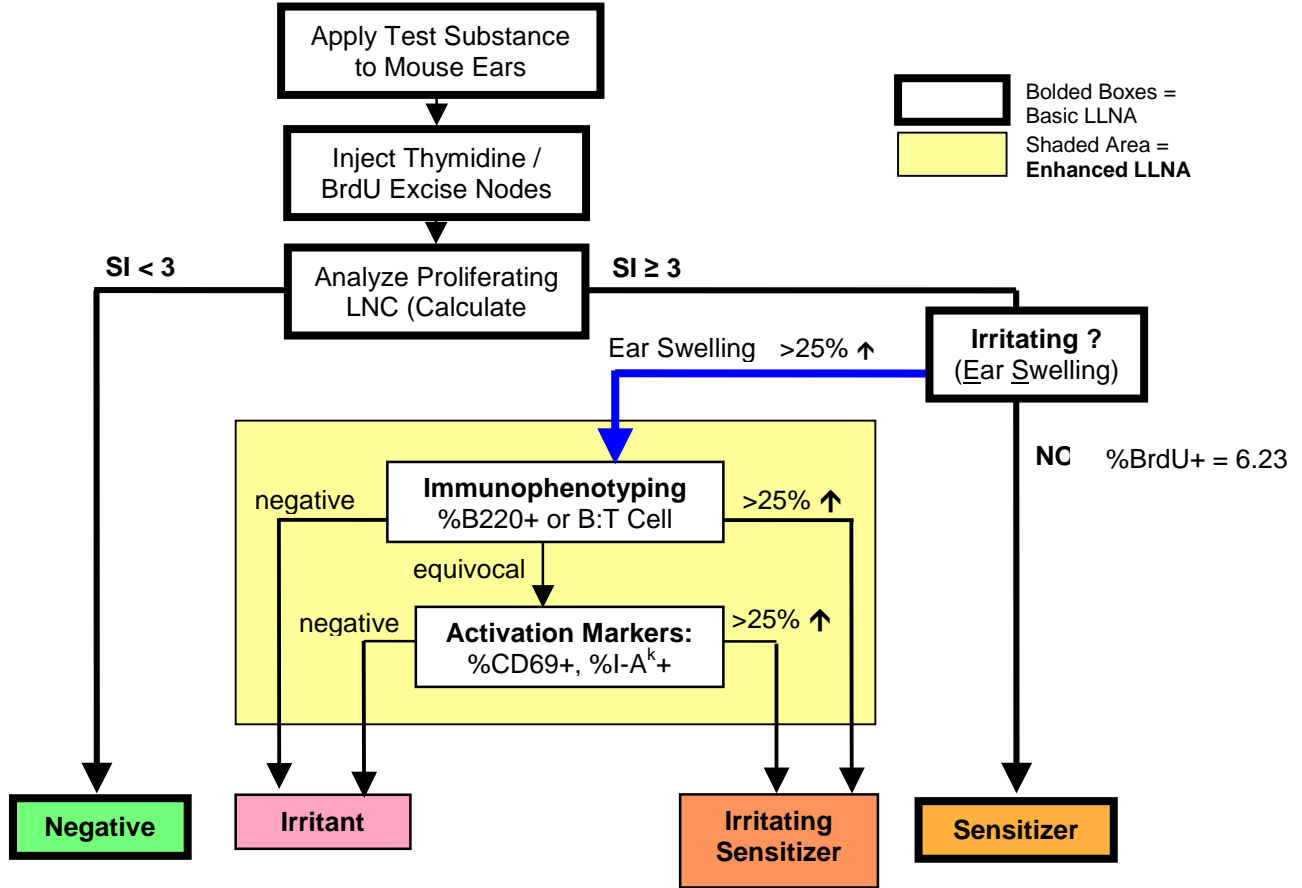


Figure 2

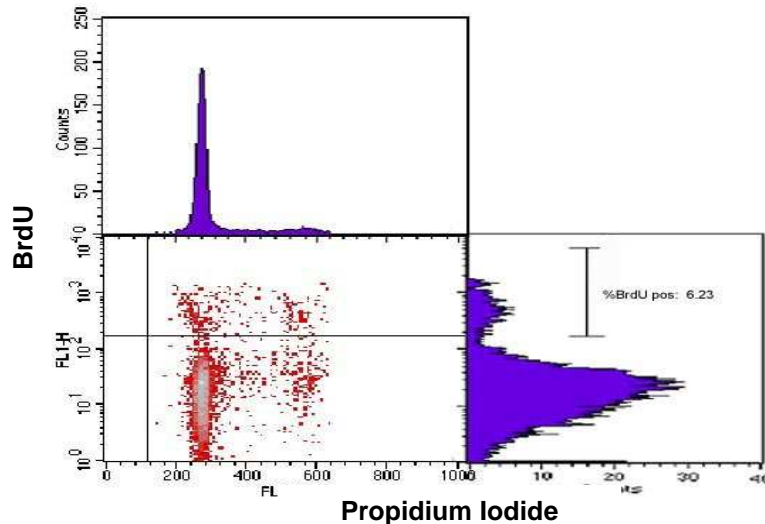
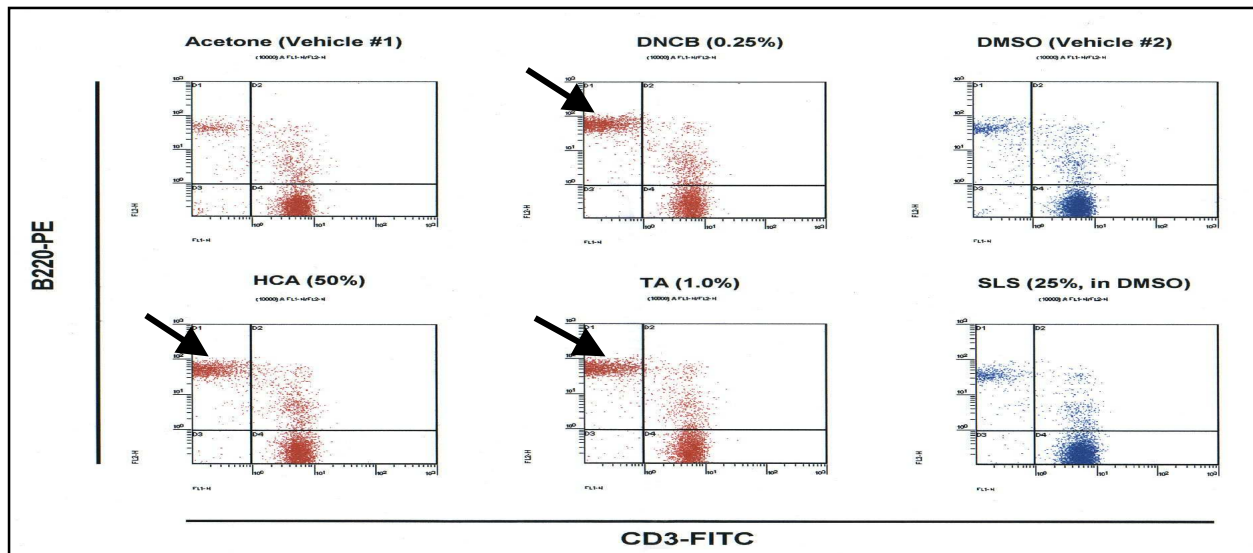


Figure 3

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

Table 1
Comparison of EC₃ 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%	0.03%
	2,4-DNCB	Strong	0.03 - 0.09% ^{a,b}	0.01 - 0.09%
	TCSA	Strong	0.04 - 0.17% ^b	0.06%
	p-Phenylenediamine	Strong	0.06%	0.45%
	Formaldehyde	Moderate	0.35% ^a	0.29%
	HCA	Moderate	7.0 - 12.2% ^c	6.3%
	Citral	Weak	13% ^a	2.0%
	Eugenol	Weak	13% ^a	13.2%
	Linalool	Very Weak	30% ^a	31.0%
	Ethylene glycol dimethacrylate	Very Weak	35% ^a	40.0%
	Isopropyl myristate	Very Weak	44% ^a	12.1%
	Propyl paraben	Very Weak	>50% ^a	>50%
Benzocaine	Very Weak	>25% ^b	>50%	
Non-Sensitizer (Irritant)	Benzalconium chloride	Irritant	>2.5% ^b	0.4%
	SLS	Irritant	6.9 - 17.1% ^b	4.84%
	Ethylenediamine	Irritant	4.4 - 10.7% ^b	2.7%
Non-Sensitizer (Non-Irritant)	Hexane	Non-Sensitizer	>100% ^a	>100%
	Sulfanilamide	Non-Sensitizer	>50% ^b	>50%
	PABA	Non-Sensitizer	>10%	16.9%
	Isopropanol	Non-Sensitizer	>50% ^b	>50%

^a = HSE contract research report 399, 2001. Development of the Local Lymph Node Assay for Risk Assessment of Chemicals and Formulations, R.J. Dearman and I. 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.

Use Of An Enhanced Local Lymph Node Assay To Correctly Classify Irritants And False Positive Substances

Figure 4

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

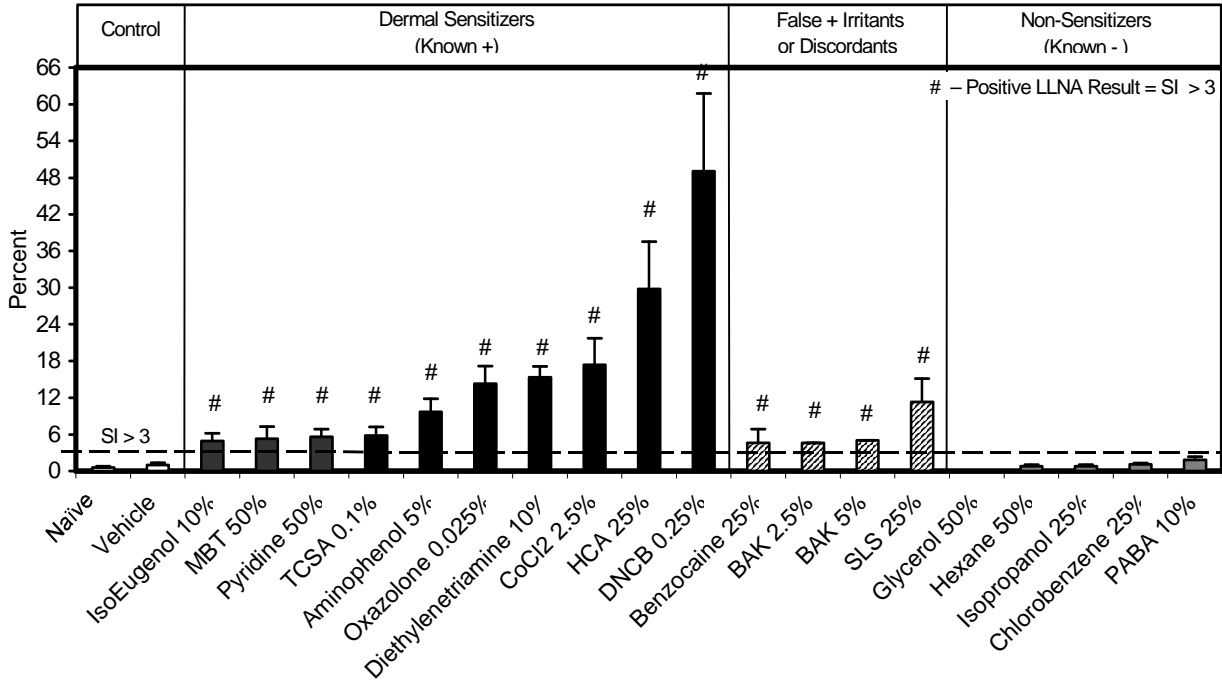
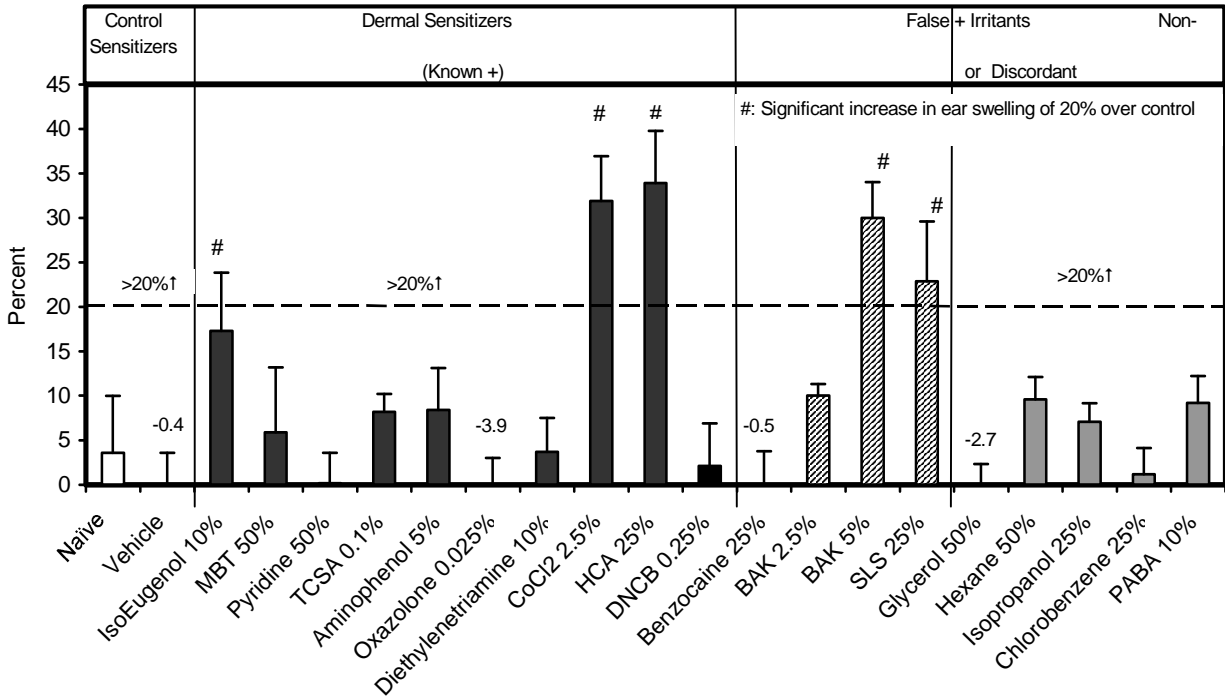


Figure 5

Ear Swelling: Percent Changes over Pre-Test Values

Figure 3. Ear Swelling: Percent Changes over Pre-Test Values



Use Of An Enhanced Local Lymph Node Assay To Correctly Classify Irritants And False Positive Substances

Figure 6

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

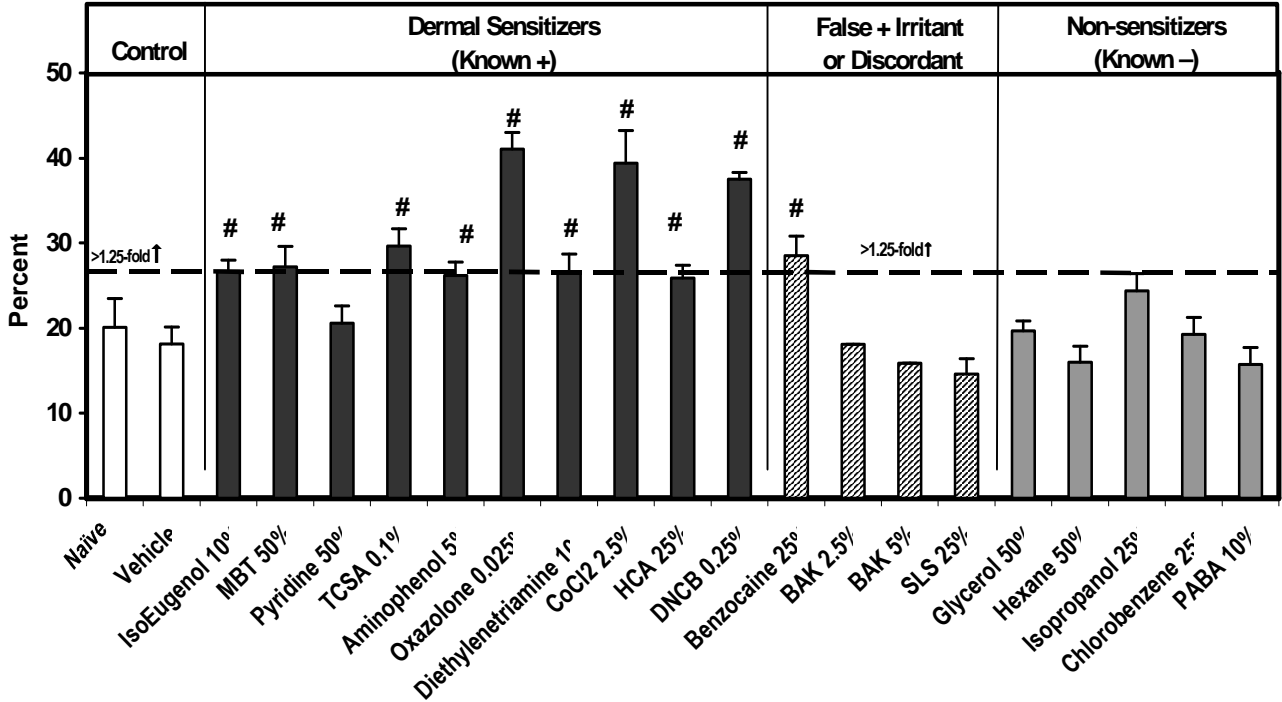
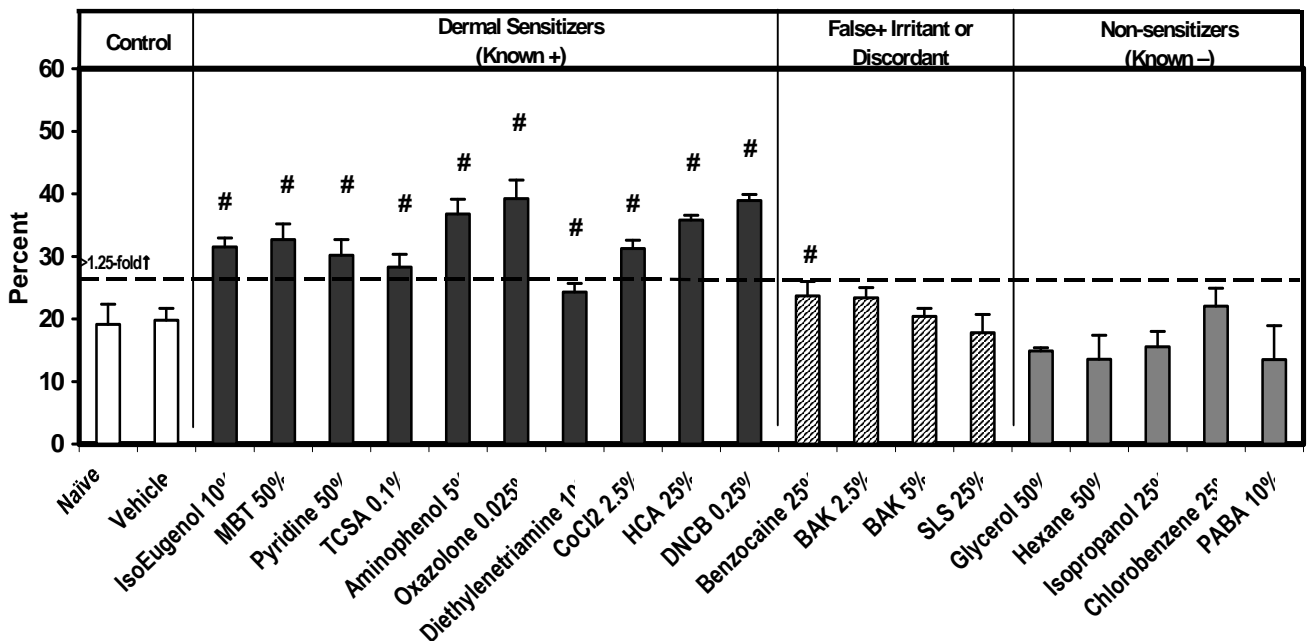


Figure 7

Percent %I-Ak+ Cell Subset in Lymph Nodes



#: Biologically significant increase of 1.25-fold over control

Table 2
Dermal Sensitization Comparison by Test Method

Positive by Radioactive LLNA	FC	R	G	H	Negative by Radioactive LLNA	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	-	-	-	-
Diphenylcyclopropanone	+	+			Nickel chloride	-	-	+	
Ethylene glycol dimethacrylate	+	+	-	+	<i>p</i> -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	+/-	+/-	+/-	+
<i>p</i> -phenylenediamine	+	+	+	+	Ethylenediamine	+	+/-	+	+
Pyridine	+	+		+	MBT	+/-	+	+	+
Sodium lauryl sulfate	+	+	-	-	Salicylic acid	+/-	-	-	-
Tetrachlorosalicylanilide	+	+	+	+					
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

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	70%	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

* = 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 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). 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 predictivity and 88% negative predictivity. By using the added immunophenotyping (B cell and T cell subtypes) immunoactivation marker (I-A^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 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 %IA^{k+} in nodal cells are useful endpoints for discriminating false positive irritants from true sensitization responses.

The project described was supported by grant number R44-ES10234-02 from the National Institute of Environmental Health Sciences (NIEHS), NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

Special thanks to BD Pharmingen, San Jose, CA for collaboration on this and other research projects at MB Research Laboratories aimed expanding the use of flow cytometry in alternative toxicology methods.