

Use of an *Ex Vivo* Local Lymph Node Assay to Assess Contact Hypersensitivity Potential

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The local lymph node assay (LLNA) is used to assess the contact hypersensitivity potential of compounds. In the standard assay, mice are treated topically with test compound to the dorsum of both ears on Days 1–3. The induction of a hypersensitivity response is assessed on Day 6 by injecting [³H]-thymidine into a tail vein and measuring thymidine incorporation into DNA of lymph node cells draining the ears. The *ex vivo* LLNA is conducted similarly except lymphocyte proliferation is assessed after *in vitro* incubation of lymph node cells with [³H]-thymidine, which significantly reduces the amount of radioactive waste. The current study tested the use of this approach for hazard assessment of contact hypersensitivity and to estimate allergenic potency. Female BALB/c mice were treated on Days 1–3 with two nonsensitizers (4'-methoxyacetophenone, diethyl phthalate), three weak sensitizers (hydroxycitronellal, eugenol, citral), one weak-to-moderate sensitizer (hexylcinnamic aldehyde), two moderate sensitizers (isoeugenol, phenyl benzoate), and one strong sensitizer (dinitrochlorobenzene). On Day 6, isolated lymph node cells were incubated overnight with [³H]-thymidine and thymidine incorporation was measured by liquid scintillation spectrophotometry. The *ex vivo* LLNA accurately distinguished the contact sensitizers from the nonsensitizing chemicals, and correctly ranked the relative potency of the compounds tested. The EC₃ values, i.e., the effective concentration of test substance needed to induce a stimulation index of 3, were as follows: 4'-methoxyacetophenone (>50%), diethyl phthalate (>50%), hydroxycitronellal (20.4%), eugenol (13.6%), citral (8.9%), isoeugenol (3.8%), hexylcinnamic aldehyde (2.7%), phenyl benzoate (2%), and dinitrochlorobenzene (0.02%). In addition, low inter-animal and inter-experiment variability was seen with 25% hexyl-cinnamic aldehyde (assay positive control). The results of the *ex vivo* LLNA in the current study were consistent with published reports using the standard LLNA and provided further

evidence that supports the use of this alternative approach to assess the skin sensitization potential of test compounds.

Keywords LLNA, topical, skin sensitization, BALB/c mice

INTRODUCTION

The murine local lymph node assay (LLNA) is recognized by the United States Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a stand-alone method for hazard identification of contact hypersensitivity (NIEHS, 1999). This assay also has been used in risk assessment to estimate the relative allergenic potency of chemicals (Basketter et al., 2000, 2001; Gerberick et al., 2001). In the standard LLNA, mice are treated topically with test compound to the dorsum of both ears for 3 consecutive days. After approximately 72 hr, mice are then given an intravenous injection of [³H]-thymidine into a tail vein to measure the proliferative response of lymphocytes in the draining lymph nodes. Based on this approach, an inherent limitation of the standard assay is the disposal of radioisotope-contaminated carcasses.

In the ICCVAM report (NIEHS, 1999), panel members commented that further investigations on alternative approaches to the standard LLNA were warranted to reduce the amount of radioactive waste. Specifically, the report recommended that additional research was needed to evaluate *ex vivo* [³H]-thymidine incorporation as one possible alternative to injecting mice with radioisotope. The use of alternative approaches to the standard assay also is supported in published guidance documents. The Organization for Economic Cooperation and Development (OECD) and the Committee for Medicinal Products for Human Use (CHMP), formally the Committee for Proprietary Medicinal Products (CPMP), both support the use of alternative endpoints in the LLNA to assess lymph node cell proliferation provided there is appropriate scientific justification that the

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alternate method yields reproducible and reliable results (CPMP, 2001; OECD, 2002).

Several reports have been published describing the development and use of alternative methods to evaluate lymphocyte proliferation in the LLNA, including *ex vivo* [^3H]-thymidine incorporation and assessment of non-radioactive approaches such as *in vivo* bromodeoxyuridine (BrdU) incorporation (van Och et al., 2000; De Jong et al., 2002; Takeyoshi et al., 2004, 2005; van den berg et al., 2005). We initially investigated the sensitivity of *ex vivo* [^3H]-thymidine incorporation by testing the contact sensitizers hexylcinnamic aldehyde and oxazolone, and the nonsensitizer methyl salicylate (Piccotti et al., 2006). Hexylcinnamic aldehyde is one of the positive controls recommended by the OECD (OECD, 2002) and has been shown to be a suitable calibrant for evaluating assay sensitivity and performance (Dearman et al., 2001). The results of these experiments demonstrated that the *ex vivo* LLNA was more sensitive than evaluating lymph node weight, lymph node cellularity and *in vitro* BrdU incorporation (Piccotti et al., 2006). Furthermore, results of the *ex vivo* LLNA with oxazolone and hexylcinnamic aldehyde were comparable to the standard assay in a direct comparison and readily distinguished the relative allergenic potencies of these compounds. This study also showed low inter-animal variability in replicate assays testing 25% hexylcinnamic aldehyde. The current study tested additional compounds to further validate the *ex vivo* LLNA for hazard assessment of contact hypersensitivity. In addition, this study assessed the ability of the assay to estimate allergenic potency by comparing calculated EC₃ values, i.e., the effective concentration of test substance needed to induce a stimulation index (SI) of 3.

METHODS

Chemicals

The test substances used in this study are listed in Table 1, and include nonsensitizing agents and chemicals classified as weak, moderate and strong contact sensitizers in the LLNA (Basketter et al., 1999; Ryan et al., 2000; Basketter and Kimber, 2001; Gerberick et al., 2001). All chemicals were prepared in acetone for dosing. Acetone has been shown to be a suitable alternative to 4:1 acetone:olive oil (AOO) in the LLNA (Gerberick et al., 1999, 2002; Woolhiser et al., 2000; Piccotti et al., 2006). Test substances and acetone were purchased from Sigma-Aldrich (St. Louis, MO), except for hydroxycitronellal, which was purchased from Acros Organics (Morris Plains, NJ).

Ex Vivo LLNA

Female BALB/c mice were purchased from Charles River Laboratories (Kingston, NY). Animals were ≈ 8 –10 weeks of age at study initiation. Mice (4/group) were treated by topical application with 25 μl of test substance in acetone to the dorsum of both ears on Days 1–3 using a single channel pipetter.

TABLE 1
Compounds tested in the *ex vivo* local lymph node assay

Compound	Standard LLNA Potency Classification ^a	<i>Ex Vivo</i> LLNA Concentrations (%)
4'-Methoxyacetophenone	Nonsensitizer	5, 10, 25, 50
Diethyl phthalate	Nonsensitizer	5, 10, 25, 50
Hydroxycitronellal	Weak	5, 10, 25, 50
Eugenol	Weak	2.5, 5, 10, 25
Citral	Weak	2.5, 5, 10, 25
Hexylcinnamic aldehyde	Weak to Moderate	2.5, 5, 10, 25
Isoeugenol	Moderate	0.5, 1, 2.5, 5
Phenyl benzoate	Moderate	0.5, 1, 2.5, 5, 10
Dinitrochlorobenzene	Strong	0.01, 0.025, 0.05, 0.1

^aPotency classifications in the standard LLNA were based on Basketter et al., 1999; Ryan et al., 2000; Basketter and Kimber, 2001; and Gerberick et al., 2001.

The concentrations tested for each compound listed in Table 1 were based on published reports (Basketter et al., 1994, 1999; Loveless et al., 1996; Ryan et al., 2000). Control animals were treated with vehicle (acetone) alone. Each experiment also included 25% hexylcinnamic aldehyde (HCA) as a positive control. Mice were observed for signs of toxicity predose and ≈ 2 –4 hr post-dose on scheduled dosing days, once daily on nondosing days, and at end of the study. Erythema on the ears was graded by visual inspection as mild (pink), moderate (red), or marked (beet red).

On Day 6, mice were anesthetized with isoflurane gas and exsanguinated. The auricular lymph nodes draining the ears were removed and placed in DMEM (Invitrogen Corporation, Grand Island, NY) supplemented with 2% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT), 1 mM sodium pyruvate (Invitrogen), 100 U/ml penicillin (Invitrogen), 0.1 mg/ml streptomycin (Invitrogen), 10 mM HEPES (Invitrogen), 0.56 mM L-arginine (Sigma), 14 μM folic acid (Sigma), 0.27 mM asparagine (Sigma), 1.5 mM L-glutamine (Invitrogen) and 50 μM 2-mercaptoethanol (Sigma).

Lymph nodes from individual animals were processed into single-cell suspensions and cell numbers were determined on a Bayer Advia 120 hematology analyzer (Tarrytown, NY). Cell density was adjusted to 1×10^6 cells/ml and 200 μl of the cell suspensions were then added to a 96-well U-bottom plate (Corning Inc., Corning, NY). Cells were incubated with 0.5 μCi /well of [methyl- ^3H]-thymidine (Amersham Biosciences, Piscataway, NJ) for 16–18 hr at 37°C under 10% CO₂. Thymidine incorporation into DNA was measured using a PerkinElmer Tri-Carb 2900TR liquid scintillation analyzer (Wellesley, MA). Data are reported as disintegrations per minute (dpm)/animal and SI. The SI was calculated by dividing the dpm of compound-treated mice by the mean of the vehicle control animals. As with the standard LLNA, a test substance that induced a SI of ≥ 3 in the *ex vivo* LLNA was considered a positive sensitizer.

The EC3 value was calculated by linear interpolation using the formula listed below to estimate allergenic potency (Basketter et al., 2000). Data points lying above a SI of 3 on the dose-response curve had the coordinate (a, b), while data points lying below a SI of 3 had the coordinate (c, d).

$$\text{Formula : EC3} = c + [(3 - d)/(b - d)] \times (a - c)$$

The sensitization potential of the compounds tested was classified as a nonsensitizer (SI < 3 at the highest concentration tested), weak (EC3 ≥ 10%), moderate (EC3 ≥ 1%, but < 10%) or strong (EC3 < 1%). These potency categories are consistent with published reports (Basketter et al., 1999; Gerberick et al., 2004).

Statistical Analysis

Treatment comparisons of lymph node cell proliferation (³H]-thymidine incorporation) were performed on rank-transformed data using a dose-trend test within one-factor analysis of variance (ANOVA). Statistical analysis was conducted separately for each compound. A pairwise test was used to evaluate the effects of the positive control, 25% hexylcinnamic aldehyde, compared to control.

RESULTS

Hazard Identification of Contact Hypersensitivity Using an *Ex Vivo* LLNA

No visible signs of irritation on the ears were seen with mice dosed 4'-methoxyacetophenone, diethyl phthalate, hydroxycitronellal, eugenol, isoeugenol, phenyl benzoate or dinitrochlorobenzene. Citral caused mild irritation on the ears at concentrations ≥ 10%, while hexylcinnamic aldehyde caused mild irritation at all concentrations tested (Table 2).

Table 3 summarizes the results of the compounds tested in the *ex vivo* LLNA. As anticipated, the nonsensitizers, 4'-methoxyacetophenone and diethyl phthalate, tested negative at concentrations ≤ 50%. The dpm values for 4'-methoxyacetophenone at concentrations of 25 and 50%, and diethyl phthalate at a concentration of 50%, achieved statistical significance; however, the stimulation indices (SIs) at these concentrations were ≤ 2. The weak sensitizers tested in this study were hydroxycitronellal, eugenol and citral. Concentration-related increases in lymphocyte proliferation were seen with hydroxycitronellal and citral. The SIs for hydroxycitronellal were 3.4 and 4.6 at concentrations of 25% and 50%, respectively. Eugenol was negative at concentrations ≤ 10%; however, treatment of mice with eugenol at a concentration of 25% induced a SI of 7.2. Citral induced SIs of 3.3 and 8.0 at concentrations of 10 and 25%, respectively.

Concentration-related increases in proliferation were seen with hexylcinnamic aldehyde (Table 3). The SIs were greater than 3 at concentrations of ≥ 5%. Treatment of mice with the

TABLE 2
Evaluation of the irritancy potential of test compounds

Compound	Clinical Observation
4'-Methoxyacetophenone	NE
Diethyl phthalate	NE
Hydroxycitronellal	NE
Eugenol	NE
Citral	Mild erythema at [10%] (Days 2-3) and at [25%] (Days 1-4)
Hexylcinnamic aldehyde	Mild erythema at [2.5-10%] (Days 1-3) and at [25%] (Days 1-5)
Isoeugenol	NE
Phenyl benzoate	NE
Dinitrochlorobenzene	NE

Mice were observed daily for clinical signs predose and approximately 2–4 hr post-dose on scheduled dosing days, once daily on nondosing days and at end of the study. Erythema on the ears was graded as mild (pink), moderate (red) or marked (beet red) by visual inspection.

NE, no drug effects observed at concentrations tested.

moderate sensitizer isoeugenol resulted in SIs < 3 at 0.5%, 1% and 2.5%; however, an SI of 3.7 was seen at a concentration of 5%. These findings were consistent with reported results for isoeugenol using the standard LLNA (Basketter et al., 1999). In an initial experiment, phenyl benzoate was tested at concentrations of 0.5, 1, 2.5 and 5%. Proliferative responses were minimal, with SIs of ≤ 2 at all concentrations tested. Based on these unexpected findings, phenyl benzoate was retested up to a concentration of 10%. In this repeated experiment, SIs > 3 were seen with phenyl benzoate at concentrations of ≥ 2.5%; however, the proliferative responses seen were not concentration-related. The SIs ranged from 3.5–3.9 at concentrations of 2.5–10%. As anticipated, treatment of mice with the strong sensitizer dinitrochlorobenzene induced concentration-related increases in SI values, with positive SIs at concentrations ≥ 0.025%.

To evaluate animal-to-animal and experiment-to-experiment variability, the proliferative responses to 25% hexylcinnamic aldehyde in 10 independent experiments were evaluated (Table 4). The coefficient of variability (CV) for 9 of 10 experiments range from 10–27%. In one assay (Experiment No. 10), the CV was 31%. The experiment-to-experiment variability over a 4–5-mo period was 44%. These results demonstrated good inter-animal and inter-experiment variability in response to hexylcinnamic aldehyde.

Use of an *Ex Vivo* LLNA to Estimate Allergenic Potency

The results shown in Table 3 demonstrated that an *ex vivo* LLNA could be used for hazard identification of contact hypersensitivity. To evaluate the use of this assay to estimate the allergenic potency of the compounds tested, EC3 values were

TABLE 3
Evaluation of contact sensitization using an *ex vivo* local lymph node assay

Chemical	Concentration (%)	DPM	SI	Chemical	Concentration (%)	DPM	SI
4'-Methoxyacetophenone	0	1445 ± 375	1.0	Hexylcinnamic aldehyde	0	818 ± 151	1.0
	5	1601 ± 254	1.1		2.5	2320 ± 506*	2.9
	10	1699 ± 281	1.2		5	3301 ± 657**	4.1
	25	1972 ± 171*	1.4		10	4902 ± 1893**	6.0
	50	2867 ± 838**	2.0		25	8029 ± 2120**	9.8
Diethyl phthalate	0	1374 ± 362	1.0	Isoeugenol	0	1029 ± 103	1.0
	5	1643 ± 209	1.2		0.5	1608 ± 417*	1.6
	10	1613 ± 283	1.2		1	2091 ± 648**	2.0
	25	1753 ± 162	1.3		2.5	2385 ± 281**	2.3
	50	1996 ± 346**	1.5		5	3795 ± 444**	3.7
Hydroxycitronellal	0	614 ± 163	1.0	Phenyl benzoate (Exp 1)	0	872 ± 212	1.0
	5	893 ± 175*	1.5		0.5	1181 ± 94	1.3
	10	1269 ± 365**	2.1		1	1322 ± 205*	1.5
	25	2064 ± 168**	3.4		2.5	1726 ± 316**	2.0
	50	2803 ± 1150**	4.6		5	1744 ± 672**	2.0
Eugenol	0	1027 ± 259	1.0	Phenyl benzoate (Exp 2)	0	702 ± 111	1.0
	2.5	1336 ± 273	1.3		1	837 ± 324	1.2
	5	1488 ± 420*	1.5		2.5	2712 ± 1338**	3.9
	10	1763 ± 172**	1.7		5	2469 ± 382**	3.5
	25	7412 ± 2171**	7.2		10	2539 ± 1046**	3.6
Citral	0	922 ± 109	1.0	Dinitrochlorobenzene	0	977 ± 188	1.0
	2.5	1435 ± 406*	1.6		0.01	1498 ± 391	1.5
	5	1840 ± 384**	2.0		0.025	4327 ± 1272**	4.4
	10	2983 ± 714**	3.3		0.05	6975 ± 1611**	7.2
	25	7367 ± 2176**	8.0		0.1	7572 ± 1265**	7.8

BALB/c mice were treated with test substance or vehicle (acetone) to the dorsum of both ears on Days 1–3. On Day 6, the auricular lymph nodes were removed and single cell suspensions were prepared. Lymph node cells were incubated *in vitro* with [³H]-thymidine for 16–18 hr and thymidine incorporation into DNA was measured by liquid scintillation spectrophotometry. Data are expressed as mean ± standard deviation, with 4 animals/group.

*Mean value statistically different than control mean, **p* < 0.05, ***p* < 0.01.

calculated and compared to published reports using the standard LLNA (Table 5). The ranking of the relative potencies of compounds tested based on the EC3 values from most-to-least potent was: dinitrochlorobenzene >>> phenyl benzoate ≅ hexylcinnamic aldehyde ≅ isoeugenol > citral > eugenol > hydroxycitronellal > diethyl phthalate = 4'-methoxyacetophenone. The EC3 values calculated using the *ex vivo* LLNA were consistent with published reports (Table 5). In addition, the estimated allergic potency of the chemicals tested in the *ex vivo* LLNA with exception to citral were consistent with the standard LLNA (Table 6). Citral is a weak sensitizer in the standard LLNA (Basketter and Kimber, 2001; Gerberick et al., 2001); however, this compound would be classified as a moderate sensitizer in this study based on its EC3 value.

DISCUSSION

The murine LLNA is used to evaluate the skin sensitization potential of compounds following topical application (Dearman et al., 1999; NIEHS, 1999; Gerberick et al., 2000). One

disadvantage of the standard assay, which relies on injection of mice with [³H]-thymidine to assess lymphocyte proliferation, is the disposal of radioisotope-contaminated carcasses. The *ex vivo* LLNA tested in the current study differs from the standard assay by incubating lymph node cells obtained from control and compound-treated animals with [³H]-thymidine *in vitro*, thus significantly reducing the amount of radioactive waste and eliminating the need for tail vein injections, which can be a source of error in the standard assay. Another difference in the assay used in this study compared to the standard LLNA is the use of BALB/c mice instead of CBA mice. We selected BALB/c mice when initiating assay development due to the limited availability of CBA mice in the United States. Importantly, BALB/c mice have been shown to be a suitable alternative to CBA mice in the LLNA (Woolhiser et al., 2000; De Jong et al., 2002; Ehling et al., 2005). One potential advantage of using an albino strain such as BALB/c mice is the ability to evaluate erythema on the ears by visual inspection, which is more difficult with pigmented CBA mice. This information can be used to help interpret the findings of the LLNA, in that concentrations that

TABLE 4
Inter-animal and inter-experiment variability in the
proliferative responses to hexylcinnamic aldehyde

Experiment ^a	Mean SI $\pm$ S.D.	Coefficient of Variation
1	7.1 $\pm$ 1.7	24%
2	4.7 $\pm$ 0.8	17%
3	8.9 $\pm$ 2.2	25%
4	14.5 $\pm$ 2.0	14%
5	9.8 $\pm$ 2.6	27%
6	5.9 $\pm$ 1.2	20%
7	4.9 $\pm$ 0.5	10%
8	7.5 $\pm$ 1.4	19%
9	10.5 $\pm$ 2.5	24%
10	14.3 $\pm$ 4.4	31%
1–10 (Inter-Experiment)	8.8 $\pm$ 3.9	44%

In Experiments 1–4 and 6–10, 25% hexylcinnamic aldehyde (4 mice/group) served as a positive control for each assay. The dose-response of hexylcinnamic aldehyde was evaluated in Experiment 5. The SI of 25% HCA is shown.

^aData shown for each experiment were used to evaluate animal-to-animal variability in the proliferative response induced by hexylcinnamic aldehyde.

induce lymphocyte proliferation but also cause visible irritation on the ears should be interpreted with caution to avoid potential false positives. Typically, non-irritating concentrations are tested in the LLNA. However, in some instances, separating ir-

TABLE 5
Comparison of calculated EC3 values from the *Ex Vivo* LLNA
to the standard assay

Chemical	<i>Ex Vivo</i> LLNA EC3 (%)	Standard LLNA EC3 (%) ^a
4'-Methoxyacetophenone	>50 ^b	>50 ^b
Diethyl phthalate	>50 ^b	NC ^c
Hydroxycitronellal	20.4	20.0–23.0
Eugenol	13.6	5.8–14.5
Citral	8.9	13.0
Hexylcinnamic aldehyde	2.7	4.4–11.5
Isoeugenol	3.8	1.3–3.3
Phenyl benzoate (Exp 1)	>5.0 ^b	1.2
Phenyl benzoate (Exp 2)	2.0	1.2
Dinitrochlorobenzene	0.02	0.03–0.08

The EC3 values for the *ex vivo* LLNA were calculated from data shown in Table 3.

^aPublished data (Basketter et al., 1994, 1999; Loveless et al., 1996; Ryan et al., 2000; Basketter and Kimber, 2001; Gerberick et al., 2004).

^bHighest concentration tested.

^cNot calculated (100% tested negative).

ritation from allergenicity may be difficult (e.g., with citral and hexylcinnamic aldehyde).

Another potential advantage of the *ex vivo* LLNA is the ability to evaluate additional endpoints in addition to lymphocyte proliferation with the same cell suspension. Sufficient numbers of cells can be obtained from individual mice to evaluate both [³H]-thymidine incorporation and lymphocyte subpopulations. Lymphocyte subset analysis by flow cytometry has been used to help differentiate irritation from allergenicity (Gerberick et al., 1999, 2002; Manetz and Meade, 1999). In addition, a histological correlate can be investigated, with one lymph node used for histopathologic evaluation (e.g., hematoxylin and eosin, immunohistochemistry) and a second for measuring lymphocyte proliferation.

An *ex vivo* LLNA has been used previously to assess the contact hypersensitivity potential of test compounds (van Och et al., 2000; De Jong et al., 2002; van den Berg et al., 2005; Piccotti et al., 2006). The current study further tested this assay for hazard assessment of skin sensitization. In addition, this study evaluated the potential use of this method in estimating allergenic potency. The *ex vivo* LLNA accurately predicted the relative contact hypersensitivity potential of the compounds tested, readily distinguishing the nonsensitizing agents from known skin sensitizers (Table 3). Importantly, these data and our previous findings (Piccotti et al., 2006) also demonstrated that this assay fulfills OECD and EMEA criteria of reproducibility and reliability, with acceptable animal-to-animal and experiment-to-experiment variability with hexylcinnamic aldehyde (the assay calibrant).

EC3 values for the compounds tested in the current study were consistent with values reported for the standard LLNA (Table 4), although small differences were seen with citral. The EC3 value for citral in the current study was 8.9, which would classify this compound as a moderate sensitizer. Citral was shown to be a weak sensitizer in the standard LLNA based on an EC3 of 13 (Basketter and Kimber, 2001), which is consistent with the potency of this compound in humans (Gerberick et al., 2001). It could be argued that there is not an appreciable difference between EC3 values of 8.9 and 13, and a value of 8.9 is close to the arbitrary cutoff for a weak sensitizer ($\geq 10\%$). With that said, citral caused mild irritation at concentrations of $\geq 10\%$ (Table 2), which could have falsely elevated the SI values at these concentrations. Thus, a potential limitation of the *ex vivo* LLNA, as with the standard assay, is the possibility of false-positive findings due to irritation or, in the case of citral, over-estimating the sensitization potential. Hexylcinnamic aldehyde is a weak sensitizer in humans (Basketter and Kimber, 2001); however, this compound is a weak to moderate sensitizer in the LLNA (Tables 5 and 6; Loveless et al., 1996; Basketter et al., 1999). This difference in potency classification also may be attributed to an over-estimation due to the irritancy properties of this compound. Despite the possibility of over-estimating the allergenic potency of citral and hexylcinnamic aldehyde in the current study, the *ex vivo* LLNA

TABLE 6
Estimated potency classifications in the *Ex Vivo* LLNA and Standard LLNA

Chemical	<i>Ex Vivo</i> LLNA ^a	Standard LLNA ^b
4'-Methoxyacetophenone	Nonsensitizer	Nonsensitizer
Diethyl phthalate	Nonsensitizer	Nonsensitizer
Hydroxycitronellal	Weak	Weak
Eugenol	Weak	Weak
Citral	Moderate	Weak
Hexylcinnamic aldehyde	Moderate	Weak to Moderate
Isoeugenol	Moderate	Moderate
Phenyl benzoate (Exp 2)	Moderate	Moderate
Dinitrochlorobenzene	Strong	Strong

The sensitization potential of the compounds in the *ex vivo* and standard LLNA was classified as a nonsensitizer (SI < 3 at the highest concentration tested), weak (EC3 ≥ 10%), moderate (EC3 ≥ 1%, but < 10%) or strong (EC3 < 1%).

^aBased on EC3 values calculated from data generated in this study (Table 5).

^bBased on EC3 values obtained from published data (Basketter et al., 1994, 1999; Loveless et al., 1996; Ryan et al., 2000; Basketter and Kimber, 2001; Gerberick et al., 2004).

correctly rank-ordered the allergenic potential of the compounds tested.

Two experiments were needed to obtain an EC3 value for phenyl benzoate (Table 3). Although the cause of these variable results remains unclear, one possible explanation was the use of acetone as vehicle. Although the authors are unaware of any published data on a vehicle comparison with phenyl benzoate, Basketter et al. (1999) showed more robust responses (i.e., higher SI values at similar concentrations) with this compound when tested in acetone-olive oil (4:1, v/v) than were seen in the current study using acetone alone, although similar EC3 values ultimately were obtained.

In summary, the results of the current study and others (van Och et al., 2000; Woolhiser et al., 2000; De Jong et al., 2002; Ehling, et al., 2005; Takeyoshi et al., 2004, 2005; van den Berg et al., 2005; Piccotti et al., 2006) collectively demonstrate the robustness of the murine LLNA, in that the hypersensitivity potential of compounds could be evaluated in different mouse stains and by measuring lymph node cell proliferation by multiple methods. Although additional positive and negative chemicals need to be tested in the *ex vivo* LLNA, the current study also provided some evidence indicating that this alternative approach could be used to estimate allergenic potency. Since the *ex vivo* LLNA uses the same quantitative endpoint (i.e., [³H]-thymidine incorporation) as the standard LLNA, an argument could be made that the validation work done for the standard LLNA could be considered when assessing the utility of the *ex vivo* assay. Thus it may be possible to test fewer compounds

to validate the *ex vivo* LLNA compared to the hundreds tested in the standard assay. A direct comparison of the *ex vivo* LLNA with the standard assay in future studies would help substantiate this possibility.

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