

The suitability of hexyl cinnamic aldehyde as a calibrant for the murine local lymph node assay

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The murine local lymph node assay (LLNA) for the prospective identification of contact allergens assesses skin sensitization potential as a function of proliferative activity induced in lymph nodes draining the site of topical exposure to test chemical. This method has been endorsed recently as a stand alone test for the identification of contact allergens. We have now examined the suitability of hexyl cinnamic aldehyde (HCA), a recommended positive control for skin sensitization testing, as a calibrant for comparing the consistency of LLNA responses with time, and between laboratories, and thus for the routine assessment of assay reliability. Standard LLNAs were performed with CBA strain mice in 3 independent laboratories over a period of 8 years. Dose-response curves were used to derive mathematically the EC₃ value (the estimated concentration of chemical necessary to cause a stimulation index (SI) of 3 compared with proliferation induced by concurrent vehicle controls). In each laboratory, 6 separate experiments were conducted using a single concentration of HCA (25%). Very similar stimulation indices were achieved, with mean values of 9.0, 6.5 and 6.6 recorded. A total of 10 dose-response experiments were performed independently in the 3 laboratories and these revealed that there was very little inter-laboratory, or temporal, variation in EC₃ values. These data confirm that HCA responses in the LLNA are very stable and demonstrate that HCA provides a suitable calibrant for determining assay sensitivity and performance.

Key words: contact allergy; local lymph node assay; positive controls; stability; hexyl cinnamic aldehyde. © Munksgaard, 2001.

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The murine local lymph node assay (LLNA) is a predictive test for the identification of chemicals that have the potential to cause skin sensitization and allergic contact dermatitis (1–4). Activity in this assay is measured as a function of proliferative responses induced in lymph nodes draining the site of exposure to the test chemical. The method has been evaluated thoroughly as a stand-alone test for skin sensitization and was endorsed recently by both the Interagency Co-ordinating Committee on the Validation of Alternative Methods (ICCVAM) in the USA, and by the European Centre for the Validation of Alternative Methods (ECVAM) in Europe (5, 6). In common with other test methods for sensitization hazard identification, it is necessary to demonstrate routinely the sensitivity of the assay and the competency of the testing laboratory.

In 1992, the Organization for Economic Cooperation and Development (OECD) recommended the

use of one of 3 mildly/moderately skin sensitizing chemicals (hexyl cinnamic aldehyde, HCA (principal name α -hexylcinnamaldehyde), 2-mercaptobenzothiazole or benzocaine) to be used for routine (2× yearly) assessment of the sensitivity and reliability of guinea pig test methods (7). More recently it has been proposed that, based upon considerations of relative potency, lack of other significant toxic properties and ready availability, only one of these chemicals (HCA) should be used as a positive control (8). Preliminary evidence from 1 laboratory has indicated that HCA provides a reliable standard for the LLNA (9). Thus, in a series of investigations conducted over a period of 18 months, HCA was shown to provoke consistent LLNA responses (9).

It appears therefore that HCA would be suitable for use as a positive control skin sensitizer for routine assessment of assay reliability, and as a calibrant for comparing relative assay sensitivity with

time and between laboratories. Here we have investigated further the utility of HCA for these purposes by comparing LLNA responses induced by this chemical in 3 independent laboratories over a period of 8 years. Such comparisons comprised both analysis of responses induced by a single concentration of HCA, and examination of dose responses using derived EC3 values; the latter being the concentration of chemical estimated to cause a 3× increase in activity compared with concurrent vehicle controls; the criterion for positivity in the LLNA (10, 11).

Materials and Methods

Participating laboratories

Syngenta Central Toxicology Laboratory, Macclesfield, Cheshire, UK (Laboratory A), Safety and Environmental Assurance Centre Toxicology Unit, Unilever Research, Colworth House, Sharnbrook, Bedfordshire, UK (Laboratory B) and Miami Valley Laboratories, The Procter & Gamble Company, Cincinnati, Ohio, USA (Laboratory C).

Mice

Young adult female CBA/Ca (Laboratories A and B; Harlan Olac, Bicester, Oxfordshire, UK) or CBA/JHsd mice (Laboratory C; Harlan Sprague Dawley Inc., Frederick, MD, USA), 6–12 weeks old, were used throughout these studies. Mice were housed under standard conditions. Food and tap water were available *ad libitum*.

Chemicals

Hexyl cinnamic aldehyde (HCA: principal name α -hexyl cinnamaldehyde; also known as 2(phenylmethylene)-octanol, a-n-hexyl-b-phenylacrolein, or 2-benzylideneoctanol) was 85% pure, the remainder being largely inactive hexyl cinnamic acid, was obtained from Aldrich Chemical Co. Ltd, Gillingham, UK or Milwaukee, WI, US. Dosing solutions were prepared volumetrically in 4:1 acetone:olive oil (AOO) and prepared freshly for each application.

Local lymph node assay

Standard protocol Laboratories A and B performed the local lymph node assay according to the standard protocol described previously (1). Groups of mice ($n=4$ or 5) were exposed topically on the dorsum of both ears to 25 μ l of various concentrations of HCA, or to the same volume of vehicle (AOO) alone, daily for 3 consecutive days. 5 days after the initiation of exposure, all mice were injected intra-

venously via the tail vein with 20 μ Ci of [^{3} H]methyl thymidine (^{3} H-TdR; specific activity 2 Ci/mmol; Amersham International, Amersham, UK) in 250 μ l of phosphate buffered saline (PBS). 5 h later, mice were killed and the draining auricular lymph nodes were excised and pooled for each experimental group. A single-cell suspension of lymph node cells (LNC) was prepared by gentle mechanical disaggregation through 200-mesh stainless-steel gauze. Cells were washed 2× with an excess of PBS and precipitated in 5% trichloroacetic acid (TCA) at 4°C. Approximately 12 h later, pellets were resuspended in 1 ml of 5% TCA and transferred to 10 ml of scintillation fluid (Optiphase "Hisafe3"; Wallac, Turku, Finland). Incorporation of ^{3} H-TdR was measured by β -scintillation counting as disintegrations per minute (dpm) per node for each experimental group. In each case, a stimulation index (SI) relative to the concurrent vehicle-treated control value was derived.

Modified procedure

Laboratory C used a modified protocol (12) based upon the standard method described above. For the dose-response experiments, modifications were: (1) daily treatment for 4, rather than 3, consecutive days with no rest days and injection of ^{3} H-TdR on the 5th day; and (2) analysis of lymph nodes obtained from individual mice, rather than lymph nodes pooled for each experimental group. For experiments in which a single concentration of HCA was used (25%), the only important modification was that lymph nodes obtained from individual mice, rather than lymph nodes pooled for each experimental group, were analyzed. Briefly, groups of mice ($n=5$) were treated on the dorsum of both ears with 25 μ l of various concentrations of HCA, or to the same volume of vehicle (AOO) alone, daily for 3 or 4 consecutive days. 72 or 24 h after the last treatment, respectively, all mice were injected intravenously via the tail vein with 20 μ Ci of ^{3} H-TdR (specific activity 5 Ci/mmol; Amersham Corporation, Arlington Heights, IL) in 250 μ l of PBS. 5 h later, mice were killed and the draining auricular lymph nodes were excised and pooled for each mouse. A single-cell suspension of LNC was prepared by gentle mechanical disaggregation through a nylon mesh filter (100 μ m pore size). Cells were washed twice with an excess of PBS and precipitated in 5% TCA at 4°C. Approximately 18 h later, pellets were resuspended in 1 ml of 5% TCA and transferred to 10 ml of scintillation cocktail (Ecolume, ICN Radiochemicals, Irvine CA). The tubes were rinsed with an additional 1 ml of TCA which was also added to the scintillation vial. Incorporation of ^{3} H-TdR was measured by β -scintillation counting

as disintegrations per minute (dpm) per node per individual animal. Stimulation indices for each experimental group were determined as the increase in ^3H -TdR incorporation relative to the concurrent vehicle-treated controls based on the group mean dpm.

Mathematical analysis

The estimated concentration of chemical required to induce an SI of 3 relative to concurrent vehicle-treated controls, or EC3 value, was derived by linear interpolation of dose-response data as described previously (11). The EC3 value was calculated by interpolating between 2 points on the SI axis, one immediately above, and the other immediately below, the SI value of 3. The vehicle-treated control (SI=1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the co-ordinates (a,b) and (c,d) respectively, then the EC3 value may be calculated using the following equation:

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

Statistical analysis

The statistical significance of differences in stimulation indices for the 6 independent experiments performed in each of the 3 separate laboratories using a single concentration of HCA (25% w/v) was examined by analysis of variance (ANOVA) using a Kruskal-Wallis test for non-parametric data (13).

Results

In attempting to investigate the utility of HCA as a calibrant for the LLNA, 2 types of analyses were

performed. In the 1st of these, data were collected from 3 independent laboratories in which, collectively, LLNA responses to 25% HCA had been measured over a period of more than 8 years. These data are summarized in Table 1 where, for each individual experiment, values (dpm/node) obtained with 25% HCA and the concurrent vehicle control (AOO) are displayed together with derived stimulation indices. The results reveal a high level of consistency with time for each of the 3 independent laboratories both with respect to incorporation of radiolabelled thymidine (in lymph nodes isolated from both vehicle-treated control animals and animals treated with 25% HCA) and with respect to derived stimulation indices. Furthermore, the background proliferative responses in lymph nodes derived from control vehicle-treated animals were very consistent between the 2 laboratories (A and B) which used identical protocols and mouse strains for the LLNA. Thus, values ranging from 159 to 495 dpm per node (mean value 352 dpm per node) and from 303 to 458 dpm per node (mean value 396 dpm per node) were recorded for Laboratory A and B, respectively. Incorporation of ^3H -TdR in lymph nodes isolated from mice exposed to 25% HCA also showed remarkably little variation in these 2 laboratories, with values ranging from 1639 to 4107 dpm per node (mean value 2955 dpm per node) and from 1827 to 3136 dpm per node (mean value 2503 dpm per node) observed in Laboratories A and B, respectively. In contrast, in the laboratory in which a modified protocol was used (the main modification being that lymph nodes from individual animals were analyzed rather than those pooled on an experimental group basis), the levels of proliferation recorded following exposure to vehicle or to 25% HCA were somewhat lower. Thus, for lymph nodes

Table 1. Temporal stability of LLNA responses to 25% HCA : an inter-laboratory comparison

Laboratory A				Laboratory B				Laboratory C			
experiment date	dpm/node AOO	dpm/node 25% HCA	SI	experiment date	dpm/node AOO	dpm/node 25% HCA	SI	experiment date	dpm/node AOO	dpm/node 25% HCA	SI
June 1994	495	4107	8.3	Feb. 1992	450	2684	6.0	Feb. 1999	127	1074	8.5
Feb. 1997	472	3841	8.1	Feb. 1994	321	2829	8.8	Feb. 1999	250	1495	6.0
April 1997	475	3396	7.2	April 1996	456	1827	4.0	May 1999	226	1382	6.1
May 1997	325	2539	7.8	Jan. 1997	458	3136	6.8	Nov. 1999	259	993	3.8
Oct. 1997	159	2207	13.9	Jan. 1998	303	2492	8.2	March 2000	169	1203	7.1
Nov. 1997	188	1639	8.7	Feb. 1999	387	2047	5.3	Nov. 2000	137	1092	8.0
mean	352	2955	9.0		396	2503	6.5		194	1207	6.6
SE	62	399	1.0		29	200	0.7		24	80	0.7

Groups of mice received 25 μl of 25% HCA in AOO, or an equal volume of vehicle alone, on the dorsum of both ears daily for 3 consecutive days. 5 days after the initiation of exposure, all mice were injected intravenously with PBS containing ^3H -TdR. Draining auricular lymph nodes were excised 5 h later and pooled on an experimental group (Laboratories A and B) or on an individual animal (Laboratory C) basis, a single cell suspension prepared and ^3H -TdR incorporation was measured by β -scintillation counting. Incorporation of ^3H -TdR (dpm/node and stimulation index (SI)) are displayed for each of 6 independent experiments conducted in each of the 3 different laboratories over a period of 8 years. Mean values are also shown.

Table 2. Stability of LLNA EC3 values derived for HCA: an inter-laboratory comparison

HCA concentration % w/v	Stimulation index (SI)										Mean	SE
	Lab A Expt 1	Lab B Expt 2	Lab C Expt 3	Lab A Expt 4	Lab B Expt 5	Lab A Expt 6	Lab B Expt 7	Lab A Expt 8	Lab B Expt 9	Lab A Expt 10		
1	ND	ND	ND	ND	1.5	ND	ND	ND	ND	ND	ND	
2.5	1.4	1.3	1.3	1.7	1.7	1.7	ND	1.3	ND	1.0	1.43	0.09
5	2.1	1.5	1.1	2.1	2.2	2.1	1.6	2.1	1.4	1.4	1.76	0.13
10	3.3	4.4	2.5	4.4	2.8	2.4	2.5	2.7	2.7	2.0	2.97	0.26
25	8.4	8.8	10.4	8.1	8.2	7.2	6.8	7.8	5.3	8.7	7.97	0.43
50	14.0	16.0	17.0	14.5	ND	14.1	ND	13.4	ND	11.6	14.37	0.66
EC3	8.8	7.6	11.0	7.0	10.6	11.9	11.7	10.9	11.7	12.2	10.34	0.59

Groups of mice received 25 μ l of a minimum of 3 concentrations of HCA in AOO, or an equal volume of vehicle alone, on the dorsum of both ears daily for 3 (Laboratories A and B) or 4 (Laboratory C) consecutive days. 5 (Laboratories A and B) or 4 (Laboratory C) days after the initiation of exposure, all mice were injected intravenously with PBS containing ^3H -TdR. Draining auricular lymph nodes were excised 5 h later and pooled on an experimental group (Laboratories A and B) or on an individual animal (Laboratory C) basis, a single cell suspension prepared and ^3H -TdR incorporation was measured by β -scintillation counting. Incorporation of ^3H -TdR (stimulation index (SI)) is displayed for each of 10 independent experiments conducted in the 3 different laboratories over a period of 8 years. The concentration of chemical required in each experiment to induce an SI of 3 (EC3 value) calculated using linear regression is illustrated also. Mean values are also shown.

isolated from vehicle-treated mice, values ranged from 127 to 259 dpm per node (mean value 194 dpm per node) and for lymph nodes derived from animals exposed to 25% HCA, values varied from 993 to 1495 dpm per node (mean value 1207 dpm per node). The lower levels of radiolabelled thymidine incorporation recorded in this laboratory for both vehicle- and HCA-treated animals may or may not reflect strain differences between the CBA/Ca mice used in the UK and the CBA/JHsd mice used in the USA. Despite the inter-laboratory differences in proliferative responses recorded for vehicle- and HCA-treated animals as described above, derived stimulation indices (SI) were very consistent both within and between all laboratories. In Laboratory A, SI values ranged from 7.2 to 13.9, in Laboratory B from 4.0 to 8.8 and in Laboratory C from 3.8 to 8.5. The corresponding mean values were, respectively, 9.0, 6.5 and 6.6. Comparison of the data sets of the stimulation indices obtained in the 3 independent laboratories using a Kruskal-Wallis test revealed that there were no significant inter-laboratory differences in derived SI values ($p=0.1193$).

We have described previously the use of EC3 values derived mathematically from LLNA dose responses for the purposes of potency estimation (10, 11). Such EC3 values have proven useful also for comparing LLNA performance in different laboratories (14–16). In a 2nd series of analyses, we therefore evaluated EC3 values for HCA obtained from a total of 10 separate experiments performed independently in 3 laboratories. These data are shown in Table 2. In each case a dose-response analysis was performed with application doses of HCA ranging from 1% (in 1 experiment, Expt. 5) to 50% (in 7 experiments). Derived EC3

values ranged from 7.0% HCA to 12.2% HCA; the mean value being 10.34%.

Discussion

The OECD has recommended that routine ($2\times$ yearly) assessments are made of the sensitivity of guinea pig assays and thus of the competence of testing laboratories. For this purpose, the use of 1 of 3 mild-to-moderate skin sensitizing chemicals was suggested. The last identified were HCA, 2-mercaptobenzothiazole and benzocaine. The latter chemical has since been shown to elicit only weak and variable responses in both guinea pig tests and the LLNA and, for this reason, it has been proposed that benzocaine is an inappropriate positive control for skin sensitization studies (17, 18). In practice, most laboratories have used either HCA or 2-mercaptobenzothiazole. However, a recent review of skin sensitization by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) concluded that in fact only a single chemical (HCA) need be used for routine checks (8). This conclusion was based on evidence that this chemical is readily available, lacks other significant toxic properties and has a potency suitable for probing the sensitivity of test methods (8). The data reported here endorse that view and provide clear evidence that in the LLNA HCA induces consistent responses with time and in different laboratories.

The analyses described here show remarkably little variation in either the stimulation indices provoked by a standard dose of HCA, or in the EC3 values derived from mathematical interpolation of dose responses. In the latter case, EC3 values ranged from 7.0% HCA to 12.2% HCA among 3

laboratories over a period of 8 years. In terms of changes in relative potency these differences are trivial. It has been suggested previously that small differences in EC3 values are of little significance in terms of overall sensitizing activity; 1 recent suggestion for potency classification being based on log-fold differences in EC3 values (11, 19).

Taken together, the data confirm that HCA responses in the LLNA are very stable. This confirmation, combined with the fact that the skin sensitization potency displayed by HCA is appropriate for interrogating assay sensitivity, suggest that this chemical provides a suitable calibrant for the LLNA. At a time when, following validation, this assay is finding increasing application by a growing number of laboratories it is important to identify a common basis for comparing and endorsing test performance. It is our view that the measurement of responses to HCA provides a suitable standard for this purpose.

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