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CONTACT DERMATITIS

The use of ethanol:diethylphthalate as a vehicle for the local lymph node assay

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The murine local lymph node assay (LLNA) is used for the prospective identification of contact allergens. This method is not only accepted as a stand-alone test for the identification of contact allergenic hazard but also used increasingly for the measurement of the relative potency of skin-sensitizing chemicals as an integral component of the risk assessment process. During the development and validation phases of the method, a list of standard vehicles for use in the LLNA was identified, among them being the vehicle most commonly used acetone/olive oil (4:1, AOO). We have now explored the performance in the LLNA of a non-standard vehicle, ethanol:diethyl phthalate (1:3, EtOH:DEP), that is used frequently to evaluate dermal effects of fragrance materials in both human and experimental studies. Current investigations have demonstrated that EtOH:DEP induces similar levels of background proliferative responses in lymph nodes comparable with the standard vehicle AOO. Moreover, expected levels of activity are observed when EtOH:DEP is used to deliver a known contact allergen in the LLNA. The conclusion drawn is that EtOH:DEP provides a suitable vehicle for use in the LLNA and that the approach described provides a basis for future evaluation of novel vehicles.

Key words: allergic contact dermatitis; local lymph node assay; regulatory toxicology; skin sensitization; vehicle control data. © Blackwell Munksgaard 2007.

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The local lymph node assay (LLNA) is a method increasingly used for the identification of skin-sensitization hazards (1, 2). This assay has been the subject of both national and international collaborative trials, and of comparisons with data available from other sources, including the results of human testing and clinical experience (1–4). On the basis of such evaluations and comparative analyses, the LLNA was validated by Interagency Coordinating Committee on the Validation of Alternative Methods as a stand-alone method for hazard identification (5, 6). Subsequently, the method was also endorsed by European Centre for the Validation of Alternative Methods (7), and in 2002, an Organization for Economic Cooperation and Development guideline (*Guideline No. 429; Skin Sensitization: Local Lymph Node Assay*) was adopted formally. More recently, the LLNA has also found application as a method for measuring the relative potency of skin-sensitizing chemicals and as an integral component of the risk assessment process (8–11).

The mechanistic basis for the LLNA is that sensitizing chemicals applied topically will provoke cutaneous immune responses that are characterized by the induced proliferation of lymphoid cells within draining lymph nodes (2). It is recognized that the vehicle in which a chemical is delivered to the skin surface can have an impact on the vigour of lymph node cell (LNC) proliferative responses (12–15). Although the choice of vehicle appears not to affect significantly proposed classifications of chemicals according to estimates of relative potency derived from the LLNA (11, 15), it is nevertheless appropriate to acknowledge vehicle effects when designing and interpreting skin-sensitization tests. These effects take on particular importance when data derived from such animal systems are used to guide the design of confirmatory human studies, such as the human repeated insult patch test, where ethics dictate that doses used determine a no-observed effect level for induction (16).

Against this background it is relevant to consider vehicles that may be appropriate for assessment of the skin-sensitizing potential of fragrance materials. It is known that significant exposure to fragrance materials results from hydroalcoholic

products on the skin (17). For this reason, ethanol (EtOH) and EtOH-containing vehicle systems are frequently used to evaluate dermal effects of fragrance materials in both human and experimental studies. A vehicle commonly employed is a 1:3 mixture of EtOH with diethyl phthalate (DEP), the latter being a common fragrance ingredient acting as a solvent and a fluidizer. Given that 1:3 EtOH: DEP is a frequently used vehicle for the assessment of the sensitization potential of fragrances, it is relevant to compare the performance of this mixture in the LLNA with the vehicle most commonly chosen for other chemical classes, 4:1 acetone:olive oil (AOO). This was the purpose of the investigations described here.

Materials and Methods

Mice

Young adult female CBA/Ca mice (Harlan UK, Bicester, Oxfordshire, UK), 6–12 weeks old, were used throughout these studies. Mice were housed in metal cages and food (SDS PCD pelleted diet; Special Diets Services Ltd, Witham UK) and water were available *ad libitum*. The ambient temperature was maintained at 22 ± 3°C and relative humidity was 50 ± 20% with a 12 hr light/dark cycle. All experiments were conducted under the provisions of the UK Animals (Scientific Procedures) Act, 1986.

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 (18), and was obtained from Aldrich Chemical Co. Ltd (Gillingham, UK). Vehicle preparations were AOO (4:1, v/v) and EtOH:DEP (1:3, v/v). Dosing solutions were prepared freshly for each application.

Local lymph node assay

The LLNA was performed according to the standard protocol described previously (19, 20). Groups of mice ($n = 4$) were exposed topically on the dorsum of both ears to 25 μl of various concentrations of chemical or to the same volume of vehicle alone, daily for 3 consecutive days. 5 days after the initiation of exposure, all mice were injected intravenously via the tail vein with 20 μCi of [^{3}H]methyl thymidine ([^{3}H]TdR; specific activity 2 Ci/mmol; Amersham International, Amersham, UK) in 250 μl of phosphate-buffered saline (PBS). 5 hr after intravenous injection of [^{3}H]TdR, mice were killed and the draining auricular lymph nodes were excised and pooled for each experimental group. A single-cell suspension of LNC was prepared by gentle mechanical disaggregation through a 200-mesh stainless-steel gauze. Cells were washed twice with an excess of PBS and precipitated in 5% trichloroacetic acid (TCA) at 4°C. Approximately 18 hr 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 (d.p.m.) per node for each experimental group. A stimulation index (SI) relative to the concurrent vehicle-treated control value was derived for each concentration of chemical tested.

EC3 value

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 (19). 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. If 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:

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

Data analysis

For the control data set from each laboratory, a normal distribution was best fitted. From this, the mean of the distribution was derived, as well as the 2.5%, 5%, 95% and 97.5% percentiles to aid in the identification of potential outliers. Differences between vehicles were compared using 1-way analysis of variance (ANOVA).

Results

During a period of 24 months starting in January 2004, a total of 48 LLNA were conducted using the preferred vehicle for the assay, AOO. During this same time period, 34 LLNAs were performed using a non-standard vehicle EtOH:DEP. Results from these assays were collated and also compared with historical data generated with the standard vehicle AOO. These latter data were collected in the same laboratory during the period June

1994 to June 2001 (44 assays in total). The distribution of thymidine incorporation in lymph nodes isolated from vehicle-treated mice is displayed in Fig. 1. The pattern of proliferation of LNCs derived from AOO-treated mice (2004–2006) was very similar to that recorded for lymph nodes isolated from EtOH:DEP-treated mice over the same time period. In both cases, there were very few experiments in which d.p.m. per node values of less than 300 were recorded. Values between 400 and 600 d.p.m./node were observed most frequently and values of 800 d.p.m./node were relatively uncommon. In contrast, there were differences in the pattern of thymidine incorporation for lymph nodes isolated from AOO-treated mice collated between 2004 and 2006, and those recorded between 1994 and 2001. In those LLNAs performed between 1994 and 2001, somewhat lower values were recorded. For example, approximately one-third of assays were shown to have d.p.m. per node values of less than 300, the most frequent values recorded were between 300 and 400 d.p.m./node and no values higher than 700 d.p.m./node were observed. The distribution of thymidine incorporation in these populations of vehicle-treated control was compared statistically after fitting to a normal distribution (Table 1). The ranges and the mean d.p.m. per node, were very similar for assays derived during the same time period for animals treated with either AOO or EtOH:DEP vehicle (mean d.p.m. per node:550 and 511 d.p.m./node, respectively). The mean d.p.m. per node for the AOO-treated mice derived between 1994 and 2001 was lower at 352 d.p.m./node and the range was also somewhat lower. Indeed, these historical AOO values were significantly lower than either the current AOO values or the current EtOH:DEP values ($P < 0.01$; 1-way ANOVA). In contrast, there were no significant differences between the distribution of vehicle control values for data collected from AOO- or EtOH:-DEP-exposed mice over the period 2004–2006. Low (2.5% and 5%) and high (95% and 97.5%) percentile values were calculated for each population. Again, these results illustrated that the d.p.m. per node values for the data set derived from AOO-treated animals conducted between 1994 and 2001 were lower than those observed for AOO- or EtOH:DEP-exposed mice in assays performed between 2004 and 2006.

Given that the vehicle control values for AOO have changed for LLNA data conducted in this

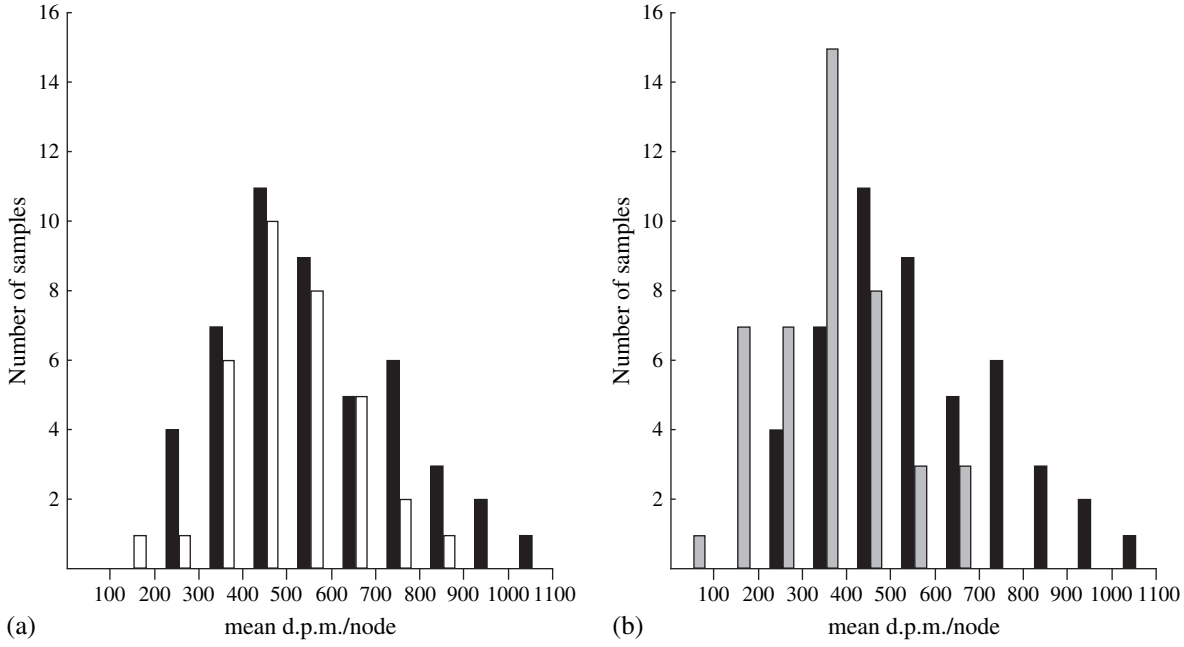

Fig. 1. Distribution of vehicle control data in the local lymph node assay (LLNA). Groups of mice received 25 μl of acetone:olive oil (AOO) or ethanol:diethyl phthalate (EtOH:DEP) on the dorsum of both ears daily for 3 consecutive days. 5 days after the initiation of exposure, all mice were injected intravenously with [^{3}H]thymidine ([^{3}H]TdR). Draining auricular lymph nodes were excised 5 hr later and pooled on an experimental group basis; a single-cell suspension was prepared and [^{3}H]TdR incorporation measured by β-scintillation counting. Mean d.p.m. per node for the vehicle control group was calculated for each experiment. **a**, Distribution of mean d.p.m. per node values derived from AOO-treated control animals in experiments conducted between January 2004 and January 2006 (■) are compared with those derived from EtOH:DEP-treated control animals in experiments conducted between January 2004 and January 2006 (□). **b**, Distribution of mean d.p.m. per node values derived from AOO-treated control animals in experiments conducted between January 2004 and January 2006 (■) are compared with those derived from AOO-treated control animals in experiments conducted between June 1994 and June 2001(▨).

Table 1. Statistical evaluation of vehicle control data in the local lymph node assay: comparison of historical and recent data[a]

Vehicle/date	*n*	Range (d.p.m./node)	Mean (d.p.m./node)	2.5%/5% percentile	95%/97.5% percentile
AOO (1994–2001)	44	97–662	352	70/115	588/634
AOO (2004–2006)	48	205–1024	550	154/217	882/945
EtOH:DEP (2004–2006)	34	196–807	511	230/275	747/799

AOO, acetone:olive oil; EtOH, ethanol; DEP, diethyl phthalate.
[a]The control data for experiments conducted between January 2004 and January 2006 using the vehicles EtOH:DEP or AOO are displayed. Historical control data for experiments conducted with AOO between June 1994 and June 2001 are also shown. All studies were carried out according to Organization for Economic Cooperation and Development Guideline 429 (22). Having fitted the range data to a normal distribution, low (2.5% and 5.0%) and high (95% and 97.5%) percentile values have been derived. Statistical significance of differences between data sets was compared by 1-way analysis of variance. $P < 0.01$, historical AOO values versus AOO (2004–2006) and historical AOO values versus EtOH:DEP (2004–2006). Not significant, AOO (2004–2006) versus EtOH:DEP (2004–2006).

laboratory between 2001 and 2004, it was relevant to determine whether this increase in control values had affected the sensitivity of the assay. We therefore compared dose responses to the material recommended as a positive control substance in tests for contact sensitization, HCA, in assays conducted between 1994–2001 and 2004–2006 (Fig. 2; 6 and 5 independent assays, respectively). Mice were exposed to AOO vehicle alone or to 5%, 10% or 25% HCA. Results are displayed as a function of SI (test d.p.m./vehicle control d.p.m.). With the exception of one experiment conducted during the period 1994–2001, where particularly vigorous responses were recorded, very similar dose responses and SI values were observed for experiments performed during 1994–2001 or during 2004–2006. The interpretation is that proliferative responses induced by sensitizers in draining lymph nodes have increased in parallel with elevated control values. Consequently, the sensitivity of the assay appears not to have been affected by the increase in background levels recorded. This is illustrated by the fact that the mean EC3 value for HCA has remained virtually unchanged; the EC3 value calculated for experiments performed between 1994 and 2001 was 10.2%, whereas the EC3 value obtained for the assays conducted between 2004 and 2006 was 10.5%.

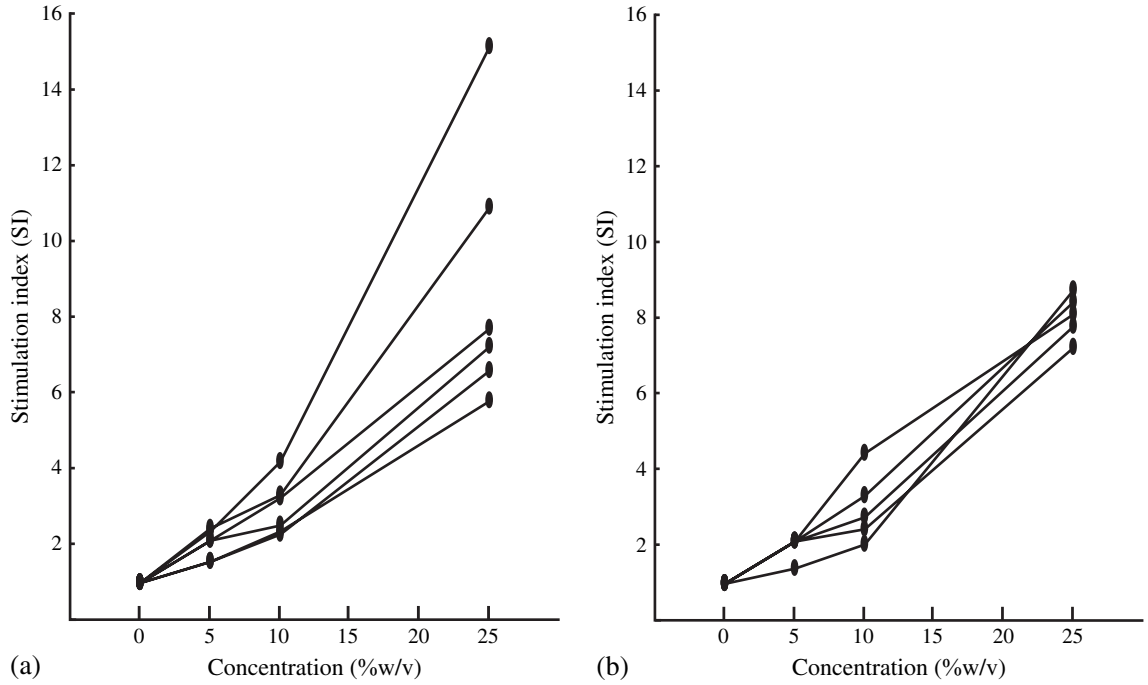

Fig. 2. Comparison of historical control and recent (2004–2006) dose-response data for hexyl cinnamic aldehyde (HCA). Groups of mice received 25 μl of various concentrations of HCA formulated in acetone:olive oil (AOO) or an equal volume of AOO vehicle alone on the dorsum of both ears daily for 3 consecutive days. 5 days after the initiation of exposure, all mice were injected intravenously with [^{3}H]thymidine ([^{3}H]TdR). Draining auricular lymph nodes were excised 5 hr later and pooled on an experimental group basis; a single-cell suspension was prepared and [^{3}H]TdR incorporation measured by β-scintillation counting. Incorporation of [^{3}H]TdR (stimulation index [SI]) is displayed for (**a**) 6 independent experiments conducted between June 1994 and June 2001 and (**b**) 5 independent experiments conducted between January 2004 and January 2006. The horizontal dotted line represents the threshold for a positive response in the local lymph node assay, an SI of 3.

Finally, dose-response experiments were conducted using the vehicle EtOH:DEP and HCA in order to determine if this non-standard vehicle had an impact on the sensitivity of the assay (Table 2). Two independent experiments were performed, with test concentrations of HCA ranging from 1% to 25% w/v. Dose-response profiles very similar to those induced by HCA formulated in AOO were observed (cf. Fig. 2), with maximal SIs of 5.3 and 6.2, and EC3 values of 15.4% and 12.4% were recorded.

Discussion

It is well established that the characteristics of the vehicle in which chemical is encountered at skin surfaces is one of the important variables that can affect the extent to which contact sensitization is acquired (12–15). For this reason, attention has been paid during the development and evaluation of the LLNA to the identification of vehicles that are appropriate for use with this test system (1). However, with increasing and more widespread use of the LLNA, it has become apparent that there is benefit in embracing a greater variety of vehicles that may confer advantages in certain circumstances. A case in point is the use of EtOH:DEP for assessment of the skin-sensitizing activity of fragrance materials. Clearly, it is necessary in such cases to provide some reassurance that use of a new and different vehicle does not introduce interpretive difficulties or affect adversely the sensitivity/selectivity of the LLNA. The need for such reassurance regarding EtOH:DEP was the basis for the investigations described here, and for this purpose, comparisons were made between EtOH:DEP and AOO, a standard and commonly used vehicle in the LLNA.

The results summarized here show clearly that, with respect to levels of background turnover by draining LNCs, EtOH:DEP does not differ from AOO during the same period of experimentation. Moreover, the standard contact allergen HCA was found to elicit LLNA responses of comparable magnitude when formulated in either AOO or EtOH:DEP. In summary, therefore, it has been shown that EtOH:DEP does not elicit unwanted increases in background proliferative responses in draining lymph nodes and is associated with expected levels of activity when used to deliver a known contact allergen in the LLNA. The interpretation is that EtOH:DEP provides a suitable vehicle for use in the LLNA.

These observations prompted us to consider in parallel the extent to which the performance of a standard vehicle (in this case AOO) in the LLNA changes with time. To this end, comparisons were made between background levels of cell turnover obtained with AOO during two separate periods of experimentation within a single laboratory. The results indicate that there had been, with time, a significant increase in control values recorded with AOO-treated mice. The reason for this change with time is uncertain but could be attributable to one or more of a number of variables including differences in spontaneous levels of lymphocyte turnover in regional lymph nodes, variations in the source and/or purity of the olive oil used in preparation of AOO or variation based on changes in operator. In practice, however, the reasons for such changes may not be of any moment as the data also show that, despite some significant change in background levels of cell turnover associated with exposure to vehicle (AOO) alone, the SIs obtained with a known contact allergen (HCA) during the two experimental periods were comparable. The interpretation is of course that changes in background lymph node values are mirrored by similar changes in the vigour of responses to skin sensitizers, such that overall there is no significant impact on SIs.

Table 2. Activity of hexyl cinnamic aldehyde (HCA) formulated in ethanol:diethyl phthalate (EtOH:DEP) in the local lymph node assay[a]

Concentration (% w/v)	Experiment 1 d.p.m./node	Experiment 1 SI	Experiment 2 d.p.m./node	Experiment 2 SI
0	346	1.0	222	1.0
1	356	1.0	340	1.5
2.5	378	1.1	247	1.1
5	559	1.6	447	2.0
10	578	1.7	541	2.4
25	1839	5.3	1374	6.2
EC3 value		15.4%		12.4%

[a]Groups of mice received 25 μl of various concentrations of HCA formulated in EtOH:DEP, or an equal volume of EtOH:DEP 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 [^{3}H]thymidine ([^{3}H]TdR). Draining auricular lymph nodes were excised 5 hr later and pooled on an experimental group basis; a single-cell suspension was prepared and [^{3}H]TdR incorporation measured by β-scintillation counting. Incorporation of [^{3}H]TdR is displayed for 2 independent experiments as mean d.p.m. per node and stimulation index (SI). EC3 values (estimated concentration of chemical necessary to elicit a threshold SI of 3) are displayed.

Taken together, therefore, it is possible to draw the following conclusions from these investigations. First, EtOH:DEP is suitable for use as a vehicle in the LLNA. Second, the approach described here provides a basis for future evaluation of the suitability of novel vehicles for use in the LLNA. Third, there may be some variation within background levels of activity in the LLNA, even in the same laboratory, but in this particular case such changes appear not to affect the performance of the assay and the vigour of responses elicited by contact allergens. It should be noted that there are circumstances in which unusual control values (abnormally high or abnormally low values) may affect interpretation of LLNA data (21). It is therefore prudent for each laboratory that conducts the assay to monitor changes in levels of thymidine incorporation in vehicle-treated control animals, and to consider on an individual assay basis whether control values fall outside the normal range for that laboratory and that are therefore potentially suspect.

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