

Human potency predictions for aldehydes using the local lymph node assay

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The murine local lymph node assay (LLNA) assesses skin sensitization potential as a function of proliferative responses induced in lymph nodes draining the site of topical exposure to test chemical. It has been shown that interpolation of LLNA dose-response data to define the concentration of test chemical required to induce a 3-fold stimulation of proliferation (EC3) offers the prospect of a quantitative index of the relative potency of a contact allergen. Initial studies have demonstrated that there exists a strong (inverse) correlation between EC3 values and contact allergenic potency in humans. Thus, materials with a low EC3 value were more potent contact allergens in humans. However, it is necessary to examine a wide range of allergens to demonstrate that such correlations are generally true. Thus, in the present study, 10 aldehydes of varying degrees of allergenicity in man were evaluated in the LLNA and their EC3 values derived. Formaldehyde was regarded as the strongest allergen in man and also had the lowest EC3 value, 0.35% (equivalent to 0.93% formalin). In contrast, the extremely weak allergen vanillin and the non-sensitizer ethyl vanillin both had EC3 values of >50%. For the remaining 7 aldehydes, there was a close similarity between what is judged to be their rank order of allergenicity in humans and EC3 values derived from analysis of LLNA data. These results support further the utility of EC3 determinations in the LLNA as a measure of the relative potency of a contact allergen.

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Many chemicals possess the inherent ability to cause skin sensitization in susceptible subjects, although this hazard does not necessarily translate into a risk of allergic contact dermatitis in humans because the level of exposure may be insufficient (1, 2). However, the incidence of the clinical expression of skin sensitization, allergic contact dermatitis (ACD), is still rather high, particularly for certain types of chemical allergens (3–5). Since hazard identification methods for contact allergens are recognized to be very successful (6, 7), a primary reason for the occurrence of ACD may be inadequacies in the risk assessment/risk management of known allergenic hazards (8, 9). The local lymph node assay (LLNA) is a modern predictive test for the identification of chemicals that have the potential to cause skin sensitization and thus allergic contact dermatitis (reviewed in (10, 11)).

Activity in this assay is measured as a function of proliferative responses induced in lymph nodes draining the site of topical exposure to the test chemical. The LLNA has been evaluated as an alternative (stand-alone) test for skin sensitization hazard identification 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 (12, 13).

In the LLNA protocol, the impact of a range of doses of chemical on proliferative activity in lymph nodes draining the site of application compared with concurrent vehicle treated controls is measured. A chemical is regarded as a skin sensitizer when, at 1 or more test concentrations, a stimulation of proliferation ≥ 3 is induced. However, it is

possible also to interpolate the dose-response curve to estimate mathematically the concentration of the chemical necessary to stimulate a 3-fold increase in proliferation (the EC3 value), and it has been proposed that this might provide a quantitative index of contact allergenic potency (14). Subsequently, the utility of EC3 values has been investigated further and these have been shown to correlate strongly with the potency of a diverse, although as yet somewhat modest, range of human contact allergens (15–19). In reality, it will be necessary to examine a diverse and substantial range of allergens to demonstrate that such correlations are generally true. In the present investigations, we have evaluated 10 aldehydes and estimated their EC3 values in a standard vehicle. These data have been used to rank the potency of the aldehydes and this ranking has been compared with what is known of their ability to cause ACD in humans.

Materials and Methods

Mice

Young adult female CBA/Ca mice (Harlan Olac, Bicester, Oxfordshire, UK) 6–12 weeks old, were used throughout these studies. Mice were housed under standard conditions. Food and tap water were available ad libitum.

Chemicals

Hexylcinnamic aldehyde (85% pure, the remainder being largely inactive hexylcinnamic acid), cinnamic aldehyde (99%), phenylacetaldehyde (85%) and vanillin (97%) were obtained from Aldrich Chemical Co. Ltd, Gillingham, UK. Ethyl vanillin (97%) was obtained from Fisher Scientific, Loughborough, Leicestershire. Formalin (37% formaldehyde) was supplied by Fisons, Loughborough, UK. Cyclamen aldehyde (100% assumed), p-tert-butyl- α -methyl hydrocinnamal (Lilial; 100% assumed) and hydroxycitronellal (100% assumed) were obtained from S. Black (Import & Export) Ltd, Hertford, Herts. p-Methylhydrocinnamic aldehyde (98%) was a generous gift from Firmenich, Geneva, Switzerland. Dosing solutions were prepared volumetrically in 4:1 acetone:olive oil (AOO) and prepared freshly for each application.

Local lymph node assay

The LLNA was performed according to the standard protocol described previously (1). Groups of mice ($n=4$) were exposed topically on the dorsum of both ears to 25 μ l of various concentrations of the test aldehyde, 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 intravenously via the tail vein with 20 μ Ci of [^{3} H]methyl thymidine (^{3} H-TdR; specific activity 2 Ci.mmol $^{-1}$; 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 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 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 disintegration 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.

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 (20). 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:

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

Results

Initially, it was necessary to classify the various aldehydes according to their relative ability to cause skin sensitization in humans (Table 1). Formalin, phenylacetaldehyde and cinnamic aldehyde were judged to be the most active allergens in this set and were placed in the moderate category, largely on the basis on data from predictive human studies (summarized previously in 18, 19). A further 5 of the aldehydes were regarded as weak human contact allergens; p-methylhydrocinnamic aldehyde and hydroxycitronellal were classified as such on the basis of predictive human test data (18, 19).

Table 1. LLNA-derived EC3 values for 10 aldehydes tested

Aldehyde	EC3 value (%)	Human potency ranking
formaldehyde	0.35 (=0.93% formalin)	moderate
phenylacetaldehyde	3.0	moderate
cinnamic aldehyde (cinnamal)	3.1	moderate
p-methylhydrocinnamic aldehyde	13.7	weak
hexylcinnamic aldehyde	14.7	weak
p-tert-Butyl- α -methyl hydrocinnamal	18.7	weak
cyclamen aldehyde	22.3	weak
hydroxycitronellal	33.0	weak
vanillin	>50.0	extremely weak
ethyl vanillin	>50.0	non-sensitizer

For the other 3 aldehydes in this “weak” category (hexylcinnamic aldehyde, p-tert-butyl- α -methyl hydrocinnamal and cyclamen aldehyde), the judgement was made largely on the basis of the clinical evidence of limited sensitization (21–24) and in the light of our understanding of the extent of use/skin exposure to the chemicals. For hexylcinnamic aldehyde, this included its close structural similarity to amylcinnamic aldehyde, a component of the standard fragrance mix, recognized as a weak human contact allergen (24, 25). In addition, hexylcinnamic aldehyde was previously placed in the same human potency category as hydroxycitronellal in an earlier publication (18). In the case of cyclamen aldehyde, there is also data from a human maximization test indicating weak sensitizing activity (26). Vanillin is a very widely used material, rarely associated with ACD and accordingly was considered to be an extremely weak allergen (27). Despite the fact that it is used commercially, no evidence could be found that ethyl vanillin had caused ACD and it was thus regarded as a non-sensitizer (28). These judgements on relative human sensitizing potency were consistent with what is understood of the chemistry of skin sensitizing aldehydes (29).

The raw dose-response data obtained for all the aldehydes tested are presented in Table 2. As reported previously, a relatively narrow range of proliferative responses was recorded for control (vehicle-treated) lymph nodes, ranging from 112 to 611 dpm node⁻¹. Topical application of the most potent of the aldehydes (formalin, phenylacetaldehyde and cinnamic aldehyde) provoked the most marked proliferative responses, with maximal SI values of 8.59, 18.96 and 15.75 achieved, respectively. Significant, although generally less vigorous, levels of proliferation were recorded for lymph nodes derived from animals treated with 1 or more concentrations of 5 of the remaining aldehydes (cyclamen aldehyde, hydroxycitronellal, p-tert-butyl- α -methyl hydrocinnamal, hexyl cinnamic aldehyde and p-methylhydroxycinnamic aldehyde).

Topical exposure to vanillin, however, despite using concentrations of up to 50%, failed to stimulate marked incorporation of thymidine. Treatment with ethyl vanillin also failed to provoke a positive LLNA response; indeed, application of increasing concentrations of this material resulted in somewhat decreased proliferation compared with vehicle-treated controls, indicative of some local or systemic toxicity at high doses.

From this dataset, the EC3 values were derived for all 10 aldehydes; these are presented in Table 1. Formaldehyde was found to be the most potent allergen, with an EC3 of 0.35% (0.93% formalin). 2 other aldehydes (phenylacetaldehyde and cinnamic aldehyde) had somewhat lower, but rather similar, EC3 values of approximately 3%. Of the remaining 7 aldehydes, 5 were relatively weak allergens, with EC3 values ranging from 13.7% to 33%, while 2 of the aldehydes, vanillin and ethyl vanillin, appeared to be essentially non-sensitizing, with EC3 values of >50%. These values compare well with those published previously for some of these aldehydes in the same vehicle: EC3 values of 2.0% were recorded for cinnamic aldehyde, 8.0% for hexylcinnamic aldehyde and 13% for hydroxycitronellal (18). The rank order of potency obtained, based on the derived EC3 values (in acetone/olive oil vehicle), was compared with human potency data (also summarized in Table 1). The EC3 data from the LLNA predicted accurately the class of skin sensitizer to which 9 of 10 of the aldehydes were assigned. The exception, vanillin, was not recognised by the LLNA as an extremely weak allergen, as it failed to generate proliferation in excess of 3-fold compared with controls at any concentration.

Discussion

Successful methods for the identification of skin sensitization hazards have been widely available for decades (6, 7). While these have been very

Table 2. LLNA dose-response data for 10 aldehydes tested

Aldehyde concentration (%w/v)	FA	PA	CA	MHCA	HCA	BMHCA	CYA	HC	EV	V
	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI	(dpm node ⁻¹) SI
0	611	255	259	285	317	112	145	351	259	302
0.1	594	—	—	—	—	—	—	—	—	—
0.5	1165	—	356	—	—	—	—	—	—	—
1	1940	185	238	—	—	—	—	—	—	—
2.5	—	450	481	355	309	145	204	770	168	264
5	3197	2000	1834	389	317	276	195	351	272	429
10	5280	2233	4087	743	563	—	—	289	193	447
25	—	4834	—	1200	1789	226	267	378	93	371
50	—	—	—	3049	—	416	47	2498	74	420
				10.69		9.26	5.16	7.11	0.29	1.39

FA, formalin; PA, phenylacetaldehyde; CA, cinnamic aldehyde; MHCA, p-methylhydrocinnamic aldehyde; HCA, hexylcinnamic aldehyde; BMHCA, p-tert-butyl- α -methyl hydrocinnamal; CYA, cyclamen aldehyde; HC, hydroxycitronellal; EV, ethyl vanillin; V, vanillin. (—=not done). dpm node⁻¹=disintegrations per minute per node; SI=stimulation index of test compared to vehicle control.

largely successful in identifying chemicals that possess a significant potential to cause sensitisation, they have been far from optimal in the provision of robust quantitative information on the potency of an allergen (8, 9). This is rather unfortunate as such data are vital for the accurate risk assessments that are necessary if potentially allergenic chemicals are to be used safely (8, 14, 15). One likely consequence of the paucity of such information is the relative prevalence of ACD from a range of chemicals, arising from the difficulties of making proper risk-assessments and associated risk-management decisions (3–5, 9, 30).

With the development of the LLNA, an opportunity arose to make an objective and quantitative measure of allergen potency, the EC3 value (14, 20). It has been clearly demonstrated that this measure is stable within (31) and between (32) laboratories for a range of allergens. EC3 values are also stable over extended periods of time (33). However the EC3 value for an allergen is of little use by itself; rather, it has to be compared with the value for other allergens and these comparisons shown to be relevant to humans. Previously, we have demonstrated that relative potency rankings in the LLNA for groups of structurally related allergens correlated with human data/evidence (16, 17). Furthermore EC3 data on 20 unrelated chemicals correlated very closely to the human evidence for classification of those materials into 1 of 5 different categories (18). This has been supplemented recently by an analysis of 21 chemicals (some of which overlapped with the first cohort of 20 substances) for which human no-effect levels for the induction of sensitization could be determined; again very strong correlations with LLNA EC3 data were obtained (19). Ultimately, it will be necessary to examine a wide range of allergens to demonstrate that such correlations are generally true.

In the present study, a series of 10 aldehydes was categorized according to what is known of their relative skin sensitizing potency in man. None of the aldehydes that were selected for this study fell into the very strongest category of skin allergens, where EC3 values typically are <0.1% (18, 19). Thus, in these investigations, only 4 categories were represented, from non-sensitizing to moderately sensitizing. EC3 data from the LLNA identified correctly the category into which each aldehyde was assigned with respect to clinical evidence. The only exception was vanillin, which appeared to be non-sensitizing in the assay, but is in fact considered to be an extremely weak human allergen; despite extensive skin contact, cases of ACD are very rarely identified. In considering these classifications, it is noteworthy that only chemicals

regarded in the LLNA as in the highest 3 classes (with EC3 values of <30% in AOO vehicle) would be classified formally as skin sensitizers under European legislation (18). Such a decision would also be consistent with the WHO position on classification of allergens (34). However, it should be noted that no formal regulatory view on how to assign contact allergens into potency classifications has been agreed. The approaches we have used here and have published elsewhere represent options which may be worthy of consideration for this purpose (18). A pragmatic solution for regulatory purposes might simply be to regard allergens with an EC3 value of <0.1% as strong, <10.0% as moderate and 10% to 30% as weak. Great care needs to be exercised, however, as it has been demonstrated that EC3 values depend on the vehicle in which skin contact occurs (reviewed in 35). This represents a very valuable tool for risk assessment, but is knowledge which adds a degree of (previously ignored) complexity to hazard identification. Although simple hazard identification was not materially affected by the use of different vehicles for the chemicals assessed, for some substances EC3 potency estimations varied by more than an order of magnitude (35).

In conclusion, the results presented in this paper demonstrate further that the LLNA provides quantitative information on skin sensitizing potency which is predictive of allergenic potency in humans. Such data promises to be a valuable tool for hazard characterization and for risk assessment.

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