Development of the local lymph node assay for risk assessment of chemicals and formulations

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Development of the local lymph node assay for risk assessment of chemicals and formulations

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Allergic contact dermatitis is a common and important occupational health problem. Although there is available a number of predictive test methods for the identification of chemicals that have the potential to cause skin sensitization, traditional assays are inappropriate for assessment of relative potency. The overall aim of this project was to examine the utility of an approach to potency assessment based on the murine local lymph node assay (LLNA), a validated method for the identification of skin sensitization hazard in which activity is measured as a function of induced proliferative responses in the lymph nodes draining the site of topical application of material. In the current investigations we have utilised an extended dose response LLNA protocol which allows the mathematical derivation of the concentration of chemical required to induce a threshold positive response (EC3 value; the effective concentration for stimulation of a 3-fold increase in lymph node cell proliferation). Using where possible chemicals for which there exist data on human exposure and contact sensitization, correlations have been made between activity in the LLNA and apparent risk to man. The influence of vehicle and formulation upon EC3 values has been examined also. Collectively, these data demonstrate that the LLNA and derived EC3 values provide a robust and reliable approach to the determination of skin sensitization potency as a first step in the risk assessment process.

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CONTENTS

SUMMARY v

INTRODUCTION 1

PROJECT AIMS 3

METHODS 5

RESULTS 7
  Inter-laboratory and temporal stability of EC3 values 7
  Correlation between EC3 values and the relative potency of contact allergens in humans 7
  Influence of vehicle and formulation on LLNA responses, skin sensitizing potential and EC3 values 8

DISCUSSION 11
  Inter-laboratory and temporal stability of EC3 values 11
  Correlation between EC3 values and the relative potency of contact allergens in humans 11
  Influence of vehicle and formulation on LLNA responses, skin sensitizing potential and EC3 values 13

CONCLUSIONS 15

REFERENCES 17

PUBLICATIONS 21

PUBLICATIONS ABSTRACTS 23

APPENDICES 25
SUMMARY

Allergic contact dermatitis is a common and important occupational health problem. Although there is available a number of predictive test methods for the identification of chemicals that have the potential to cause skin sensitization, traditional assays are inappropriate for assessment of relative potency. The overall aim of this project was to examine the utility of an approach to potency assessment using the murine local lymph node assay (LLNA), a validated method for the identification of contact allergens in which activity is measured as a function of induced proliferative responses in the lymph nodes draining the site of topical application of material. Those chemicals that induce a stimulation index of three or more compared with concurrent vehicle-treated controls are considered to have the potential to cause skin sensitization and the assay is now used routinely in many laboratories for the identification of sensitization hazard. In the current investigations we have utilized an extended dose response LLNA protocol which allows the mathematical derivation of the concentration of chemical required to induce a stimulation index of three (EC3 value). Using where possible chemicals for which there exist data on human exposure and contact sensitization, correlations can then be made between the activity in the LLNA and apparent risk to man. We have demonstrated the temporal and inter-laboratory stability of LLNA responses in the context of derived EC3 values and determined the most appropriate mathematical approach for the calculation of EC3 values from LLNA dose response data. We have examined also the influence of vehicle and formulation upon EC3 values and sensitizing potency and the correlation between EC3 values and the relative potency of contact allergens in humans. Collectively, these data demonstrate that the LLNA and derived EC3 values provide a robust and reliable approach to the determination of skin sensitization potency as a first step in the risk assessment process.
INTRODUCTION

Skin sensitization resulting in allergic contact dermatitis is a common and important occupational health problem. Many hundreds of chemicals have been implicated as causes of contact allergy and there clearly exists a need for accurate identification of those chemicals that have the potential to induce skin sensitization.

Until recently the species of choice for the predictive assessment of skin sensitizing activity was the guinea pig. Two guinea pig test methods in particular found favour with toxicologists; the Guinea Pig Maximization Test (Magnusson and Kligman, 1970) and the Occluded Patch Test of Buehler (Buehler, 1965). More recently, an increased understanding of the immunobiological events which result in skin sensitization has created opportunities for the development of alternative test methods, including methods using mice. The most thoroughly investigated of these newer approaches is the murine local lymph node assay (LLNA; Kimber et al., 1994; Dearman et al., 1999).

Unlike guinea pig test methods, the identification of skin sensitizing chemicals in the LLNA is based upon consideration of responses induced during the induction, rather than elicitation, phase of contact hypersensitivity. Skin sensitization is achieved when a susceptible individual is exposed topically to a local concentration of the inducing contact allergen sufficient to stimulate a specific immune response; the hallmark of sensitization being T lymphocyte activation. The sequence of events can be summarized as follows. Topical encounter with a chemical allergen induces or increases the expression by epidermal cells of a variety of cytokines. Some of these are responsible for the mobilization, migration and functional maturation of epidermal Langerhans cells (LC). These cells, together possibly with other cutaneous dendritic cells, transport antigen from the skin to draining lymph nodes where it is presented to responsive T lymphocytes (Kimber and Cumberbatch, 1992; Kimber et al., 1998a; Kimber et al., 1999). Antigen-driven T cell activation results in division and differentiation; the selective clonal expansion of allergen-specific T lymphocytes representing the cellular basis of sensitization. Following subsequent exposure of the now sensitized subject to the inducing chemical allergen at the same or a different site, an accelerated and more aggressive secondary immune response will be provoked. Allergen-responsive T lymphocytes recognize and respond to allergen in the skin at the site of exposure and initiate the inflammatory response that is the clinical manifestation of allergic contact dermatitis (Basketter et al., 1999b).

The LLNA is based upon measurement of the induction by chemicals of the T lymphocyte proliferative response in skin draining lymph nodes which is necessary for the acquisition of contact sensitization. This read-out for the assay is particularly appropriate since it has been demonstrated experimentally that there exists a close correlation between the vigour of allergen-induced lymph node cell proliferative responses and the extent to which skin sensitization develops (Kimber and Dearman, 1991).

Chemicals that, at one or more concentration, induce a three-fold or greater increase in LNC proliferative activity compared with controls (induce a stimulation index [SI] of 3 or more) are classified as skin sensitizers. Chemicals which fail at all test concentrations to provoke an SI of 3 are considered to be non-sensitizers. On this basis the LLNA has been used to examine the skin sensitizing potential of over 200 chemicals and has been the subject of comparisons with guinea pig test methods and human data and of extensive national and international collaborative trials (Basketter et al., 1991; Basketter and Scholes, 1992; Kimber and Basketter, 1992; Kimber et al., 1991; 1995; 1998b; Loveless et al., 1996). Taken together the results of these investigations have shown the LLNA to be a robust and reliable method for the identification of contact allergens (reviewed in Dearman et al., 1999). On the basis of these and other data, the LLNA has been endorsed recently as a stand alone method for skin sensitization hazard detection by both the US Interagency Coordinating Committee.
on the Validation of Alternative Methods (ICCVAM) and by the European Centre for the Validation of Alternative Methods (ECVAM) (Gerberick et al., 2000).

More recently, consideration has been given to the possibility that LLNA responses induced by chemicals could be used also for determining relative skin sensitization potency as a first step in the risk assessment process. As indicated previously the vigour of LNC proliferative responses appears to correlate closely with the extent to which contact sensitization will be acquired (Kimber and Dearman, 1991). A critical determinant of the induction phase of contact sensitization is the amount of chemical allergen encountered per unit area of skin (Friedmann, 1990; 1996), thus relative skin sensitizing potency is best described as a function of the concentration of chemical necessary for the acquisition of sensitization. On this basis relative potency is determined in the LLNA by derivation of an EC3 value, this being defined as the amount of test chemical required to elicit a stimulation index of 3. In effect, therefore, the strategy is to define the intrinsic sensitizing potency of a chemical as a function of the concentration required to elicit a LLNA response of the magnitude which in practice is necessary for classification as a contact allergen. Thus, those chemicals with relatively low EC3 values are considered to be stronger skin sensitizers than are chemicals that have high EC3 values (Basketter et al., 1997; 1999a; Hilton et al., 1998). On this basis it is possible to rank the relative potency of skin sensitizing chemicals. Moreover, in the context of developing risk assessments it is possible also to compare EC3 values between the test chemical and a chemical or chemicals for which information is available regarding dose response relationships for contact sensitization in humans (Kimber and Basketter, 1997).

The potential utility of this approach for determining the relative skin sensitizing potency of chemicals was examined as part of a previous grant from the HSE (Contract No 3583/R51.128 “Local lymph node assay for risk assessment of chemicals”). During the course of this grant, the most appropriate mathematical approach for derivation of EC3 values from LLNA dose responses was determined. Three approaches were compared; quadratic regression analysis, the Richard’s model and simple linear interpolation. On the basis of studies conducted with 10 chemicals with a range of sensitization potencies it was concluded that linear interpolation between values either side of the threefold SI on a LLNA dose response curve provides the most convenient and robust method for calculating EC3 values (Basketter et al., 1999a). Preliminary investigations with known skin sensitizers of varying sensitization potency revealed that LLNA EC3 values correlated closely with what is known of skin sensitizing activity in humans and that the ability of a chemical allergen to stimulate cutaneous immune responses and to induce skin sensitization was influenced by the form in which it is encountered on the skin (Basketter et al., 1999a; Lea et al., 1999).
PROJECT AIMS

As described above, our experience to date (derived from the previous two year programme of work supported by the Health and Safety Executive through contract number 3583/R51.128) indicates that the LLNA can be used for the determination of relative skin sensitizing potential as well as for hazard identification. Such an approach, if validated, would be of great value in risk assessment and occupational health management.

The aims of the current grant are therefore to address in greater detail three main issues:

(i) a more detailed comparison of LLNA EC3 values with human skin sensitization data
(ii) a systematic examination of the impact of formulation on sensitization potential
(iii) a consideration of the most appropriate approaches to incorporation of EC3 values into risk assessments and recommendations for an integrated approach to risk assessment.
METHODS

The murine LLNA was conducted as described previously (Basketter and Kimber, 1992). Groups of CBACa strain mice (n=4) were exposed topically on the dorsum of both ears to 25µl of various concentrations of the test chemical, or to the same volume of vehicle alone, daily for three consecutive days. Five days after the initiation of exposure all mice were injected intravenously via the tail vein with 250µl of phosphate buffered saline (PBS) containing 20μCi of [³H] methyl thymidine (³HTdR; specific activity 2Ci/mmol). Five hours later the mice were killed and the draining auricular lymph nodes excised and pooled for each experimental group. Single cell suspensions of lymph node cells (LNC) were prepared by mechanical disaggregation though 200-mesh stainless steel gauze. Cells were washed twice with PBS and precipitated in 5% trichloroacetic acid (TCA) at 4°C overnight. Pellets were then resuspended in 1ml of 5% TCA and transferred to 10ml of scintillation fluid. Incorporation of ³HTdR 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 was derived.

The estimated concentration of chemical required to induce a stimulation index of 3 relative to concurrent vehicle-treated controls, or EC3 value, was derived by linear interpolation as described previously (Basketter et al., 1999a). The EC3 value was calculated by interpolating between two points on the SI axis, one immediately above, and the other immediately below, the SI value of three. The vehicle-treated control value (SI=1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of three have the co-ordinates (a,b) and (c,d) respectively, then the EC3 value may be calculated using the following equation:

\[ EC3 = c + \frac{(3-d)(a-c)}{(b-d)} \]

Where possible, the chemicals have been assigned to 5 discrete classes of sensitizing potency. These assignments have been made on the basis of the limited published literature available on human predictive testing, with either the human maximization test or the human repeat insult patch test, and expert judgement based on decades of accumulated experience of clinical contact dermatitis (Basketter et al., 2000; 2001; Gerberick et al., 2001).

Class 1 : strong allergens
Class 2 : moderate allergens
Class 3 : weak allergens
Class 4 : extremely weak (very limited sensitizing potency) allergens
Class 5 : non-sensitizers

In other cases, chemicals have been simply classified as sensitizers (Class 1-3) or non-sensitizers.
RESULTS

Inter-laboratory and temporal stability of EC3 values

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 (twice yearly) assessment of the sensitivity and reliability of guinea pig test methods (OECD, 1999). 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 (European Centre for Ecotoxicology and Toxicology of Chemicals [ECETOC], 1999). We have therefore examined the suitability of HCA 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 three independent laboratories (Syngenta Central Toxicology Laboratory, CTL; Unilever Safety and Environment Assurance Centre; Miami Valley Laboratories, Procter & Gamble) over a period of 8 years. In each laboratory, 6 separate experiments were conducted using a single concentration of HCA (25%). Very similar stimulation indices were achieved in the three independent laboratories, with SI values ranging from 7.2 to 13.9, from 4.0 to 8.8 and from 3.8 to 8.5. The corresponding mean values were, respectively, 9.0, 6.5 and 6.6. A total of 10 dose response experiments were performed independently in the three laboratories and these revealed that there was very little inter-laboratory, or temporal, variation in EC3 values. Thus, derived EC3 values ranged from 7.0% HCA to 12.2% HCA; the mean value being 10.4% (Dearman et al., 2001).

Correlation between EC3 values and the relative potency of contact allergens in humans

As outlined in the milestone plan, the sensitizing potential of more than 20 chemicals of different chemical classes and a range of sensitizing potencies has been investigated in the LLNA and EC3 values derived. LLNA responses to the chemicals listed below were investigated, and EC3 values derived, where possible, for each chemical. To facilitate between chemical comparisons, the majority of these chemicals were applied in the standard vehicle of choice, 4 : 1 acetone : olive oil (AOO).

EC3 values have been determined for 10 aldehydes of varying degrees of allergenicity in man, as assessed by a clinical dermatologist (Basketter et al., 2001). The sensitizing potencies of the aldehydes selected for these studies were in the categories of moderately sensitizing (Class 2) to non-sensitizing (Class 5); none of the aldehydes investigated were classified as strong allergens (Class 1). LLNA responses to all chemicals were examined following their application in the standard vehicle, AOO. Aldehydes identified as moderate human sensitizers (formaldehyde; phenylacetaldehyde and cinnamic aldehyde) had EC3 values in the range of 0.35-3.1%. A further 5 chemicals classed as weak sensitizers (p-methylhydrocinnamic aldehyde, hexyl cinnamic aldehyde, 4-tert-butyl-alpha-methylhydrocinnamic aldehyde, cyclamen aldehyde and hydroxycitronellal) had much higher EC3 values of between 13.5 and 33%. The remaining aldehydes vanillin and ethyl vanillin, classified as extremely weak and non-sensitizing, respectively, in each case failed to provoke a 3-fold increase in proliferation at the maximum concentration tested (50%).
Additional chemicals have been examined in the LLNA as follows:

Three known human skin sensitizers diethyl maleate (Ryan et al., 2000), propyldenephthalide (Ryan et al., 2000) and dimethylaminopropylamine (DMAPA; Wright et al., 2001), (Class 1-3 allergens; not defined) induced vigorous proliferative responses and EC3 values of 2.0%, 2.7% and 2.2% were derived, respectively. Two Class 1 allergens, methyl(chloro) isothiazolinone /methylisothiazolinone (MCI/MI), the active ingredients of the preservative Kathon® CG, and diphenylecyclopropeneone (DPC) elicited EC3 values of 0.005% and 0.05%, respectively (Basketter et al., 2000). An EC3 value of 1.3 was recorded for the Class 2 allergen isoeugenol (Basketter et al., 2000) and a considerably lower EC3 value of 26% for the Class 3 allergen methlyhexanedione (Gerberick et al., 2001).

Application of benzocaine in AOO (maximum concentration 50%) did not provoke a positive LLNA response, consistent with recent experience of this material as a very weak (Class 4) allergen (Warbrick et al., 2000). LLNA dose responses have been performed with a further 6 chemicals evaluated and classified as non-sensitizers in humans. Glycerol (which was formulated in dimethylformamide), diethyl phthalate and hexane failed to provoke an SI of 3 or more at any test concentration examined, despite employing maximum concentrations of 100% (Basketter et al., 2000). Octanoic acid, 4’methoxyacetophenone (acetanisole) and isopropanol also failed to stimulate positive LLNA responses at any concentration tested (maximum 50%) (Gerberick et al., 2001).

**Influence of vehicle and formulation on LLNA responses, skin sensitizing potential and EC3 values**

Previous investigations have revealed that the vehicle in which a chemical is administered can have significant effects on the vigour of LLNA responses and skin sensitizing activity (Lea et al., 1999). These initial experiments were performed with 1,4-hydroquinone (HQ), a sensitizer that would be classified as of moderate potency, formulated in 7 different vehicle systems each tested in 2 independent laboratories. These preliminary data demonstrated that EC3 values could vary by as much as 14-fold as a result of vehicle matrix. There was a high degree of concordance between the two independent laboratories with respect to derived EC3 values for HQ in each vehicle. The impact of vehicle and formulation on derived EC3 values and determination of skin sensitization potency has therefore been considered in greater detail.

In experiments performed in collaboration with Unilever Safety and Environment Assurance Centre, the influence of vehicle on derived EC3 values has been examined for 6 additional contact allergens : 2-mercaptobenzothiazol (MBT), MCI/MI, 3-dimethylaminopropylamine (DMAPA), isoeugenol, cinnamic aldehyde (CA) and ethylene dimethacrylate (EGDMA). Chemicals were dosed in a minimum of 6 out of the following 8 vehicles: 4:1, acetone:olive oil (AOO), methyl ethyl ketone (MEK), dimethylformamide (DMF), dimethylsulphoxide (DMSO), propylene glycol (PG), acetone, 50:50 mix of ethanol:water and a 90:10 mix of ethanol:water. These data and those described above for HQ are shown in Tables 1a and 1b.
Table 1a. Derived EC3 values for a range of chemicals : influence of vehicle matrix.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>MBT</th>
<th>HQ</th>
<th>MCI/MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetone</td>
<td>20.1</td>
<td>0.07</td>
<td>0.0076</td>
</tr>
<tr>
<td>AOO</td>
<td>&lt;2.5</td>
<td>0.11</td>
<td>0.0049</td>
</tr>
<tr>
<td>DMF</td>
<td>6.3</td>
<td>0.19</td>
<td>0.0075</td>
</tr>
<tr>
<td>DMSO</td>
<td>6.2</td>
<td>0.33</td>
<td>0.0075</td>
</tr>
<tr>
<td>MEK</td>
<td>&lt;2.5</td>
<td>0.10</td>
<td>0.0068</td>
</tr>
<tr>
<td>50:50 ETOH/H2O</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>90:10 ETOH/H2O</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PG</td>
<td>&gt;50</td>
<td>&gt;1</td>
<td>0.048</td>
</tr>
</tbody>
</table>

Vehicles used were : acetone, acetone:olive oil 4 : 1 (AOO), dimethylformamide (DMF), dimethylsulphoxide (DMSO), methylethyl ketone (MEK), 50:50 mix of ethanol:water (ETOH/H2O), a 90:10 mix of ethanol:water (ETOH/H2O) and propylene glycol (PG). Chemicals used were 2-mercaptobenzothiazol (MBT), hydroquinone (HQ) and methyl(chloro) isothiazolinone /methylisothiazolinone (MCI/MI).

Table 1b. Derived EC3 values for a range of chemicals : influence of vehicle matrix.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>DMAPA</th>
<th>isoeugenol</th>
<th>CA</th>
<th>EGDMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetone</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>20</td>
</tr>
<tr>
<td>AOO</td>
<td>2.2</td>
<td>1.0</td>
<td>1.7</td>
<td>37</td>
</tr>
<tr>
<td>DMF</td>
<td>1.7</td>
<td>1.4</td>
<td>0.5</td>
<td>32</td>
</tr>
<tr>
<td>DMSO</td>
<td>3.2</td>
<td>0.9</td>
<td>0.9</td>
<td>34</td>
</tr>
<tr>
<td>MEK</td>
<td>1.8</td>
<td>1.0</td>
<td>1.0</td>
<td>28</td>
</tr>
<tr>
<td>50:50 ETOH/H2O</td>
<td>7.1</td>
<td>4.9</td>
<td>1.2</td>
<td>ND</td>
</tr>
<tr>
<td>90:10 ETOH/H2O</td>
<td>4.1</td>
<td>1.8</td>
<td>1.6</td>
<td>ND</td>
</tr>
<tr>
<td>PG</td>
<td>&gt;10</td>
<td>2.5</td>
<td>1.4</td>
<td>15</td>
</tr>
</tbody>
</table>

Vehicles used were : acetone, acetone:olive oil 4 : 1 (AOO), dimethylformamide (DMF), dimethylsulphoxide (DMSO), methylethyl ketone (MEK), 50:50 mix of ethanol:water (ETOH/H2O), a 90:10 mix of ethanol:water (ETOH/H2O) and propylene glycol (PG). Chemicals used were 3-dimethylaminopropylamine (DMAPA), isoeugenol, cinnamic aldehyde (CA) and ethylene dimethacrylate (EGDMA).

EC3 values were determined from LLNA dose response data using linear regression. ND = not done
Data published in Lea et al., 1999; Warbrick et al., 1999a; Wright et al., 2001.

Standard LLNA dose responses were performed using concentrations based on previous experience with each chemical. Thus MCI/MI was tested using a range of concentrations from 0.00075% to 0.075%, CA was tested at 0.1% to 2.5%, isoeugenol and DMAPA were utilized at 0.5% to 10%, whereas MBT and EGDMA were tested at 2.5% to 50%.
It was found that for some chemicals (such as MBT and HQ) the vehicle in which the material was formulated had a profound effect on activity. For other chemicals (including EGDMA and CA), the vehicle matrix had little impact on derived EC3 values (ranging from 15% to 37% and 0.5% to 1.7%, respectively). For MBT, EC3 values varied up to 20-fold as a result of formulation, with AOO and MEK vehicles resulting in particularly low EC3 values (<2.5%), whereas MBT applied in PG failed to stimulate a positive LLNA response at the highest concentration tested (50%). For the sensitizers HQ, CMI/MI and DMAPA, application vehicle caused derived EC3 values to vary between 14-fold and 6-fold. For each of these three materials, the PG formulation gave the highest EC3 value, with the other vehicles giving rather similar EC3 values for each chemical. Application of isoeugenol in all of the vehicles tested resulted in positive LLNA responses, with the DMSO formulation inducing the most vigorous proliferative responses and the lowest EC3 value (0.9%). A narrow range of EC3 values was observed for isoeugenol when formulated in the other vehicles (ranging from 1.0% in MEK to 2.5% in PG) although application of this allergen in ethanol:water 50:50 resulted in somewhat less vigorous responses compared with those obtained following application in the other matrices used (EC3 value of 4.9%).

In 1992, the OECD in a revised guideline for skin sensitization testing recommended that benzocaine be used as a positive control substance. However, previous experience has indicated that both LLNA and guinea pig predictive tests conducted with this material give weak and variable results (Basketter et al., 1993; 1995). As demonstrated above, the vehicle in which a chemical is administered can have significant effects on skin sensitizing activity. LLNA responses to benzocaine were examined following formulation in 6 different vehicle matrices: AOO, DMSO, DMF, MEK, acetone:saline (1:1) and acetone. Benzocaine failed to provoke an SI of 3 or more at any concentration tested (2.5% to 50%) in any vehicle used (Warbrick et al., 2000).
DISCUSSION

Inter-laboratory and temporal stability of EC3 values

The temporal stability of LLNA responses to, and derived EC3 values for, the contact allergen HCA have been examined previously (Dearman et al., 1998). In each of 5 experiments performed over a period of 10 months in a single laboratory (Syngenta CTL), similar LLNA responses were observed and comparable EC3 values derived. In a second series of experiments, similar investigations of the stability of responses to another contact allergen, p-phenylenediamine, were conducted concurrently in two independent laboratories (Syngenta CTL and Unilever Safety and Environment Assurance Centre). These studies confirmed the stability, with time and across laboratories, of EC3 values derived from consideration of LLNA responses (Warbrick et al., 1999b).

In the subsequent studies reported herein, an inter-laboratory assessment of the stability of responses to HCA over a period of some 8 years has been undertaken. These analyses showed 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 this period of 8 years (Dearman et al., 2001). 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; one recent suggestion for potency classification being based on log-fold differences in EC3 values (Basketter et al., 1999a; 2000).

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.

Correlation between EC3 values and the relative potency of contact allergens in humans

LLNA EC3 values have been derived for a further 21 chemicals which have been assigned to one of 5 classes of human allergen. For the series of 10 aldehydes, LLNA EC3 data identified correctly the category of allergenic potency in man into which each aldehyde was assigned, with the exception of vanillin, which was identified as a non-sensitizer. In fact, vanillin is considered to be an extremely weak human allergen (Class 4); despite extensive skin contact, cases of atopic contact dermatitis are very rarely identified (Basketter et al., 2001). The two Class 1 allergens MCI/CI and diphenylcyclopropeneone were also correctly assigned in the LLNA as potent allergens with very low EC3 values (0.005% and 0.05% respectively). Isoeugenol and methylhexanediol had EC3 values orders of magnitude higher than the Class 1 allergens (1.3% and 26%, respectively), which were consistent with their classifications as Class 2 and Class 3 human allergens. Application of benzocaine in AOO (maximum concentration 50%), however, did not provoke a positive LLNA response (EC3>50%) and it was thus classified in the LLNA as a non-sensitizer. Recent experience indicates that in fact this material is a very weak allergen (Class 4), with contact allergy to benzocaine being largely associated with conditions where there is repeated application to compromised skin, such as pruritus ani (Angelini, 1992). All 6 non-sensitizers (Class 5) were identified correctly, as despite employing maximum test concentrations of 50% or 100%, none of these materials (glycerol, diethyl phthalate, hexane, octanoic acid, acetanisole or...
isopropanol) elicited a positive response in the LLNA. These data described above have been collated with previously published LLNA EC3 data from this and other collaborating laboratories (Table 2; Basketter et al., 2000; 2001; Gerberick et al., 2001).

Table 2  Comparison of LLNA EC3 values with human potency estimation

<table>
<thead>
<tr>
<th>Human Class 1</th>
<th>LLNA EC3 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>methyl (chloro) isothiazolinone / methylisothiazolinone</td>
<td>0.005%</td>
</tr>
<tr>
<td>diphencyclopropenone</td>
<td>0.05%</td>
</tr>
<tr>
<td>p-phenylenediamine</td>
<td>0.06%</td>
</tr>
<tr>
<td>2,4-dinitrochlorobenzene</td>
<td>0.08%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human Class 2</th>
<th>LLNA EC3 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>glutaraldehyde*</td>
<td>0.09%</td>
</tr>
<tr>
<td>formaldehyde</td>
<td>0.35%</td>
</tr>
<tr>
<td>isoeugenol</td>
<td>1.3%</td>
</tr>
<tr>
<td>phenacetaldehyde</td>
<td>3.0%</td>
</tr>
<tr>
<td>cinnamic aldehyde</td>
<td>3.1%</td>
</tr>
<tr>
<td>tetramethylthiuram disulphide</td>
<td>6.0%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human Class 3</th>
<th>LLNA EC3 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>citral</td>
<td>13%</td>
</tr>
<tr>
<td>eugenol</td>
<td>13%</td>
</tr>
<tr>
<td>p-methylhydrocinnamic aldehyde</td>
<td>14%</td>
</tr>
<tr>
<td>hexyl cinnamic aldehyde</td>
<td>15%</td>
</tr>
<tr>
<td>p-tert-butyl-α-methyl hydrocinnamal</td>
<td>19%</td>
</tr>
<tr>
<td>cyclamen aldehyde</td>
<td>22%</td>
</tr>
<tr>
<td>methylhexadione</td>
<td>26%</td>
</tr>
<tr>
<td>hydroxycitronellal</td>
<td>33%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human Class 4</th>
<th>LLNA EC3 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>linalool</td>
<td>30%</td>
</tr>
<tr>
<td>ethylene glycol dimethacrylate</td>
<td>35%</td>
</tr>
<tr>
<td>isopropyl myristate</td>
<td>44%</td>
</tr>
<tr>
<td>penicillin G*</td>
<td>46%</td>
</tr>
<tr>
<td>propyl paraben</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>vanillin</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>propylene glycol</td>
<td>&gt;100%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human Class 5</th>
<th>LLNA EC3 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethyl vanillin</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>octanoic acid</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>isopropanol</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>4’methoxyacetophenone (acetanisole)</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>glycerol*</td>
<td>&gt;100%</td>
</tr>
<tr>
<td>hexane</td>
<td>&gt;100%</td>
</tr>
<tr>
<td>diethyl phthalate</td>
<td>&gt;100%</td>
</tr>
</tbody>
</table>

*All materials formulated in AOO with the exception of glycerol (DMF), penicillin G (DMSO) and glutaraldehyde (acetone)
In total, 32 chemicals with allergenic potencies spanning the 5 classifications of human allergen have been examined. The most potent allergens (Class 1; CMI/MI, 2,4-dinitrochlorobenzene, dipencyclopropenone and p-phenylenediamine) all had very low LLNA EC3 values of 0.05% or less. With the exception of glutaraldehyde (EC3 = 0.09%) which is incorrectly classified by the LLNA EC3 data as a potent allergen, those chemicals assigned as moderate (Class 2) human allergens had EC3 values ranging from 0.35% (formaldehyde) to 6% (tetramethylthiuram disulphide), considerably higher values than those recorded for the potent allergens. A possible explanation for the apparent misclassification of glutaraldehyde is the fact that this EC3 value is derived from a LLNA conducted in a different vehicle to the majority of chemicals (DMF rather than AOO). All 8 contact sensitizers classified as weak human contact allergens (Class 3) were positive in the LLNA and were assigned correctly, with EC3 values ranging from 13% to 33%. Four of the 7 very weak allergens were positive in the LLNA, with EC3 values of between 30% and 46% recorded. The remaining three of these materials were negative in the LLNA, despite being tested at maximum concentrations of 50% or 100%. All of the 7 non-sensitizers (Class 5 allergens) were correctly identified as negative in the LLNA (SIs of less than 3 were recorded at all concentrations tested, including at 50% or 100%).

These data demonstrate that not only is the LLNA able to identify correctly all those allergens (Classes 1, 2 and 3) which would be formally classified as skin sensitizers under European legislation and according to the criteria of the World Health Organization (World Health Organization, 1997), but is able also to generate information regarding relative sensitization potency.

**Influence of vehicle and formulation on LLNA responses, skin sensitizing potential and EC3 values**

A series of investigations on the influence of formulation and vehicle on LLNA responses and derived EC3 values for a number of confirmed contact allergens has been performed. These data reveal that vehicle matrix can impact on EC3 values derived in the LLNA. As reported previously (Basketter et al., 2000; 2001; Gerberick et al., 2001), with respect to relative potency between chemicals, EC3 values which differ by more than 10-fold only are considered to be important, with modest changes such as those observed for EGDMA (2.5-fold) considered not to be significant. Such effects were chemical-dependent, in that for some sensitizers such as cinnamic aldehyde and EGDMA EC3 values were not affected substantially by formulation, whereas responses to sensitizers such as MBT or CMI/MI were markedly affected by the vehicle matrix. The lack of vehicle effects was not simply a function of contact sensitizing potency; for example MBT is considered to be of similar potency to cinnamic aldehyde and yet these materials differ markedly with respect to the influence of vehicle on derived EC3 values.
CONCLUSIONS

As outlined in the milestone plan, LLNA EC3 values have been derived for more than 20 chemicals each of which has been assigned to one of 5 classes of allergen based on experience of sensitization potential in man. In addition, the influence of formulation on LLNA responses has been examined for 6 different sensitizers.

Taken together, these results support strongly the use of EC3 values derived using the LLNA in the estimation of relative skin sensitization potency and the use of that information in the risk assessment process. Ranking of these materials with respect to activity in the LLNA gives an order broadly similar to that expected from the evidence available in man for sensitization potency, with orders of magnitude of differences in derived EC3 values and therefore threshold concentrations between chemicals of differing potency. Our current recommendation is that allergens with an EC3 value of less than 0.1% should be regarded as potent, those which range from 0.1% to 10% should be regarded as moderate whilst those allergens with EC3 values of 10% or more should be regarded as weak allergens. Materials which fail to elicit a positive response despite testing at high concentrations should be regarded as non-sensitizers.

The results from these investigations have revealed also that the vehicle matrix can have a profound effect on the effectiveness of skin sensitization and that in this context the LLNA may provide a useful approach for determining the influence of formulation on sensitization potency. They confirm that for the majority of chemicals, selection of vehicles from a standard panel comprising AOO, MEK, DMSO or DMF would be the most appropriate when assessing skin sensitization hazard (Kimber and Basketter, 1992). Unless an inappropriate vehicle (such as PG, for the majority of chemicals) is chosen, then vehicle effects are unlikely to impact on the sensitivity and selectivity of the LLNA for the purposes of hazard identification but should be a consideration when assigning potency classifications. Additional considerations for vehicle selection, apart from solubility, include assessment of local and systemic toxicity of any vehicle/compound compound combination.
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PUBLICATIONS (ABSTRACTS)


APPENDICES

