STUDY

Contact Allergenic Potency: Correlation of Human and Local Lymph Node Assay Data

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Background: **Effective toxicologic evaluation of skin sensitization requires that potential contact allergens are identified and that the likely risks of sensitization among exposed populations are assessed. By definition, chemicals that are classified as contact sensitizers have the capacity to cause allergic contact dermatitis (ACD) in humans. However, this hazard is not an all-or-nothing phenomenon; clear dose-response relationships can be discerned and thresholds identified for both the induction of sensitization and the elicitation of ACD. Commonly, these parameters are grouped under the heading of potency, the determination of which is vital for risk assessment. Preclinical testing for sensitization potential is critically important for hazard assessment before human exposure. The murine local lymph node assay (LLNA) is the most recently accepted test method for sensitization hazard assessment.**

Objective: **The aim was to compare potency estimations derived from LLNA data with clinical determinations of relative potency based on human data.**

Methods: **No-effect levels (NOELs) for a range of 21 chemicals were determined from nondiagnostic human repeat patch test studies as reported in the literature. These levels were compared with LLNA EC_3 values, the estimated concentration required to produce a 3-fold increase (positive response) in draining lymph node cell (LNC) proliferative activity.**

Results: **Using available human repeat patch test data, together with expert judgment, the compounds were classified as strong, moderate, weak, extremely weak, or nonsensitizing. Additionally, the potency of each chemical was classified independently based on its LLNA EC_3 value. The results show clearly that LLNA EC_3 values are very comparable with the NOELs calculated from the literature. Moreover, the potency rankings based upon LLNA EC_3 data support their human classification.**

Conclusion: **The present investigations show that the LLNA can be used to provide quantitative estimates of relative skin sensitizing potency EC_3 values that correlate closely with NOELs established from human repeat patch testing and from our clinical experience.**

FOR NEW PRODUCTS or product ingredients that come into contact with the skin, it is necessary, before their introduction on to the market, to conduct a thorough skin safety testing and risk assessment program to be certain that the product will be well tolerated. One critical risk assessment process involves the determination of allergic skin reactions, referred to as skin sensitization, the clinical manifestation of which is allergic contact dermatitis (ACD). The skin sensitization risk assessment process has been described in detail elsewhere.[1-5] Essential elements for conducting a sound risk assessment involve the development of an understanding of the sensitization potential of the contact allergen and the likely dose, nature, extent, and duration of exposure.[6] One area of difficulty in the development of a quantitative risk assessment for a contact allergen is objective information regarding its relative potency compared with other skin sensitizers.[4,6,7]

For years, it has been well known that chemical allergens display dose-response characteristics regardless of whether the sensitization is induced in an experimental system[8,9] or in humans.[10,11] Despite the importance of potency estimation in the development of accurate risk assessments, there has been relatively little progress in the identification of appropriate models. Although the standard guinea pig tests such as the maximization test have been very successful at hazard identification,[12,13] there has been some interest in the use of a modified guinea pig maximization test for consideration of relative potency. Of particular note have been the efforts of Andersen et al,[14] who have manipulated the guinea pig maximization test to obtain dose-response data. However, the development of a novel predictive assay in the mouse, the local lymph node assay (LLNA),[15-18] provides new opportunities for the objective and quantitative estimation of skin sensitization potency.[19,20] For the purposes of hazard identification, the LLNA measures sensitization potential as a function of lymphocyte proliferative responses induced in draining lymph nodes by test chemicals; those chemicals that at one or more test concentration provoke a 3-fold or greater increase in lymph node cell (LNC) proliferation compared with vehicle controls are classified as potential contact

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allergens. This method has more recently been applied to determination of relative potency, with comparisons between chemicals based on the mathematical derivation of an estimated concentration (EC_3) value, this being the estimated concentration of chemical necessary to cause a 3-fold increase in lymph node cell proliferative activity. Experience to date with this approach has been very encouraging; clear differences between skin sensitizing chemicals can be discerned and such differences appear to correlate with the ability to induce contact allergy in experimental models[21] and with what is known of their sensitizing activity among humans.[22-23] It is this latter correlation that is of greatest significance in evaluating the accuracy of relative potency determinations made using the LLNA and the utility of these in the risk assessment process.

We have shown previously that derivation of the EC_3 for a chemical provides an objective and quantitative measure of potency when compared with chemicals that had been assigned to classes based on their human sensitizing potency.[7] Twenty chemicals were assigned to 1 of 5 human potency classes (strong, moderate, weak, extremely weak, and nonsensitizing) based solely on the expert clinical judgement of the authors. These classifications correlated well with the calculated EC_3 values from LLNA studies. In this article, in contrast, we have determined the potency rankings for 21 chemicals based on quantitative data from human repeat patch test studies reported in the literature, together with our clinical experience, and compared these with the rankings derived from LLNA EC_3 values.

Materials and Methods

Estimation of Relative Skin Sensitization Potency in Man

For ethical reasons, there are no well-defined or widely applied methods for the determination of relative skin sensitization potency in humans. In consequence, we examined the published literature for reports of dose-response induction studies that have been conducted in humans, thus yielding information on the sensitizing potency of the chemical. Specifically, a review of the limited, but nevertheless valuable, published literature on nondiagnostic human repeat patch testing, including both the human maximization test (HMT)[24] and the human repeat insult patch test (HRIPT)[25-29] was conducted. To help rank order the contact allergens tested in humans, a no-effect level (NOEL) for each chemical was determined. For comparison with LLNA EC_3 values, NOELs were expressed as a function of dose per unit area of skin $\mu g/cm^2$ calculated from the concentration tested, patch size, and application volume. However, in some instances in which a true NOEL was not defined, we were limited to using either the lowest effect concentration (lowest effect level [LOEL]), or the highest concentration tested that did not give a response in on HRIPT or HMT procedure. For each of the compounds evaluated, the key references used in determining these concentrations are given in Tables 1 and 2. These data, along with expert judgement based extensive clinical experience of ACD (e.g., clinical diagnostic patch test data), were used to classify the compounds as strong, moderate,

Table 1. Comparison of Human and LLNA Data: Sensitizers

Chemical	*Human Classification*	*NOEL HRIPT ($\mu g/cm^2$)*	*NOEL HMT ($\mu g/cm^2$)*	*LLNA EC_3 ($\mu g/cm^2$)*	*LLNA Potency Classification*	*Key References*
Methylcholroisothiazolinone methylisothiazolinone	Strong	1		2.5	Strong	7,23,38,40
p-phenylenediamine	Strong	10*†		15	Strong	7,26,33
2,4-dinitrochlorobenzene	Strong	8.8†‡		20	Strong	7,10,32
Glutaraldehyde	Moderate	100*§		23§	Strong	7,22,26
Formaldehyde	Moderate	37§		162§	Moderate	22,26
Isoeugenol	Moderate	69	5,517†	450	Moderate	32
Cinnamic aldehyde	Moderate	591	345	500	Moderate	7,38,41,42
Citral	Weak	775		3,250	Weak	7,38,42,42
Eugenol	Weak	1,938	5,517	2,225	Weak	32
Hydroxycitronellal	Weak	2,953	3,448	5,000	Weak	7,42,43
5-methyl-2,3-hexanedione	Weak		3,448†	6,450	Weak	44
p-methylhydrocinnamic aldehyde	Weak		1,379	~6,250	Weak	44
Linalool	Extremely weak		13,793	7,600	Weak	44
Penicillin G	Extremely weak		17,241†	11,600	Extremely weak	24,45
Propylene glycol	Extremely weak	60,000	17,241	NC‖ 25,000¶	Nonsensitizer	24,26,46

*Assume a patch area of 5 cm^2 for the Johnson & Johnson Square Band-Aid (New Brunswick, NJ).
†Lowest level tested with positive response.
‡Induction consisted of a single patch application of 62.5 $\mu g/7.1$ cm^2. This dose sensitized 8% of the subjects tested.
§$\mu g/cm^2$ value is calculated for formaldehyde and glutaraldehyde. Test material used in study was formalin (37% formaldehyde) The test material used for glutaraldehyde was 50%.
‖NC, not calculated. No positive response was obtained at any concentration tested and, therefore, an EC_3 value could not be calculated.
¶Highest concentration tested.

Table 2. Comparison of Human and LLNA Data: Nonsensitizers

Chemical	*Human Classification*	*NOEL HRIPT ($\mu g/cm^2$)*	*NOEL HMT ($\mu g/cm^2$)*	*LLNA EC_3 ($\mu g/cm^2$)*	*LLNA Potency Classification*	*Key References*
Octanoic acid	Nonsensitizer		690†	NC, 12,500†	Nonsensitizer	46
4 methoxyacetophenone (acetanisole)	Nonsensitizer		4,138†	NC, 12,500†	Nonsensitizer	44
Isopropanol	Nonsensitizer		6,897†	NC, 12,500†	Nonsensitizer	46
Glycerol	Nonsensitizer		13,793†	NC, 25,000†	Nonsensitizer	44
Hexane	Nonsensitizer		68,966	NC*, 25,000†	Nonsensitizer	24,46
Diethylphthalate	Nonsensitizer	38,760	68,966	NC, 25,000†	Nonsensitizer	44

*NC, not calculated. No positive response was obtained at any concentration tested and, therefore, an EC_3 value could not be calculated.
†Highest concentration tested

weak, extremely weak, or nonsensitizers. The results of this exercise are contained in Tables 1 and 2.

LLNA Protocol and Chemicals Tested

The LLNA was conducted as described elsewhere.[16,18] The data reported here are derived from existing published studies. Representative references for the sources of LLNA data for each of the chemicals discussed are provided in Tables 1 and 2. However, it should be recognized that the calculated EC_3 figures have been derived from a consideration of each laboratory's experience with these chemicals.

Potency Estimation in the LLNA

The approach to the estimation of relative skin sensitization potency of chemicals in the LLNA has previously been described in detail.[16,30] It is based on the mathematical estimation of the concentration of chemical necessary to obtain a 3-fold stimulation of proliferative activity in draining lymph nodes (compared with concurrent vehicle treated controls) and is thus termed the EC_3 value. The stability of this measure has already been demonstrated,[31] as has its interlaboratory reproducibility[32,33] and stability with time.[31] Preliminary investigations also have indicated that it might represent a useful correlate of skin sensitization potency in humans.[7,22,23] In these present investigations, existing data on 21 chemicals evaluated in the LLNA have been used to derive EC_3 values. The calculation of the EC_3 values was carried out by linear interpolation according to the equation

$$EC_3 = c + [(3 - d)/(b - d)] \times (a - c)$$

in which the data points lying immediately above and below the stimulation index (SI) value of 3 on the LLNA dose response plot have the coordinates (a,b) and (c,d), respectively.[30] The calculated EC_3 values (in percentage application concentrations) were converted to $\mu g/cm^2$ values using the standard applied dose volume of 25 μL and an exposure area of 1 cm^2 so that a direct comparison could be made with the human data. Comparison of percentage levels can be misleading, because different volumes and patch areas are used in the different human test systems.[6] For the purpose of these comparisons an arbitrary classification scheme for the LLNA was used (Table 3). This was comprised of 5 classes based on EC_3 values (here defined as $\mu g/cm^2$ of the chemical calculated to induce a 3-fold increase in lymph node cell [LNC] proliferative activity compared with concurrent vehicle control). It must be emphasized that this particular classification scheme has been adopted here solely for the purpose of facilitating comparisons between LLNA data and sensitizing activity in humans. Consequently, it must be recognized that these classes, and the way in which they are defined with respect to EC_3 values, do not necessarily represent the only or best approach to classification of skin sensitizing potency as a function of LLNA data.

Results

Using the available HRIPT or HMT data together with expert judgment, the compounds were classified as strong, moderate, weak, extremely weak, or nonsensitizing. Derived LLNA EC_3 values for each of the 21 chemicals considered here are listed in Tables 1 and 2. Based on their LLNA EC_3 values, each chemical was assigned a potency classification (see Table 3). The lowest EC_3 value (highest sensitization potency) was obtained with methylchloroisothiazolinone/methylisothiazolinone 2.5 $\mu g/cm^2$ which had a strong LLNA potency classification because its EC_3 value was below 100 $\mu g/cm^2$. The HRIPT data clearly support this classification based on the NOEL which, calculated from the literature, is 1 $\mu g/cm^2$. Additionally, clinical experience would support this material's classification as a strong allergen.[6,34] Two other chemicals *p*-phenylenedi-

Table 3. LLNA Potency Classification

LLNA EC_3 ($\mu g/cm^2$)	*Potency Classification*
<100	Strong
100-1,000	Moderate
1,000-10,000	Weak
>10,000	Extremely Weak
NC*	Nonsensitizer

*NC, not calculated. No positive response is obtained at any concentration tested; therefore, an EC_3 value can not be calculated.

amine and 2,4-dinitrochlorobenzene (DNCB) were classified clinically as strong allergens based primarily on the available HRIPT data as well as our clinical experience. It is important to note that a compound's potency classification is not based solely on observed prevalence in the dermatology clinic because prevalence can be influence by how widespread the use of the ingredient is and to the extent of exposure to the ingredient. Therefore, the primary driver on potency classification is the available HRIPT data in which there is an observed NOEL. Both of these chemicals were classified also as strong in the LLNA because their EC_3 values are less than 100 $\mu g/cm^2$. The LLNA EC_3 values for each of the chemicals classified as strong are very similar to the calculated NOELs. It is important to note that the NOEL for 2,4-dinitrochlorobenzene is based on using a single occluded exposure study design and not a repeat patch test procedure.[10] Regardless of these considerations, both the human and LLNA data would support the classification of DNCB as a strong allergen.

Based on the human NOELs obtained from the literature and the clinical experience with glutaraldehyde, formaldehyde, isoeugenol, and cinnamic aldehyde, these materials were classified as moderate human sensitizers. The LLNA results for formaldehyde, isoeugenol, and cinnamic aldehyde support a moderate classification based on their EC_3 values being in the range of 100 to 1,000 $\mu g/cm^2$; however, glutaraldehye was classified as a strong allergen with an EC_3 value of 23 $\mu g/cm^2$ in the LLNA.

Five chemicals were classified as weak human allergens based on available human data as well as our clinical experience (citral, eugenol, hydroxycitronellal, 5-methyl-2,3-hexanedione, and *p*-methylhydrocinnamic aldehyde). The NOELs calculated for these materials were significantly higher than the values for allergens classified as strong or moderate. A NOEL was not available for 5-methyl-2,3-hexanedione, so the lowest concentration tested that gave a response in an HMT study is given in Table 2. Each of the 5 chemicals was classified as weak in the LLNA based on their EC_3 values, ranging from 1,000-10,000 $\mu g/cm^2$.

Three chemicals were classified as extremely weak, linalool, penicillin G, and propylene glycol, based on the available human data and clinical experience. Similar to the human classification, penicillin G is classified as extremely weak in the LLNA because its EC_3 value is over 10,000 $\mu g/cm^2$. On the other hand, an EC_3 value could not be calculated for propylene glycol in the LLNA; thus, it was classified as a nonsensitizer. Propylene glycol, reported as a rare allergen,[35] was negative in the LLNA when tested up to 100%. Linalool was given a human classification of extremely weak, whereas it was classified as weak in the LLNA based on its EC_3 potency value of 7,600 $\mu g/cm^2$.

Table 2 lists 6 chemicals classified as nonsensitizers based on the available human data and clinical experience (octanoic acid, 4-methoxyacetophenone, isopropanol, glycerol, hexane, diethylphthalate). The NOELs calculated for these materials from published HRIPT and HMT data are substantially higher than those listed for the sensitizers. In fact, hexane and diethylphthalate were negative in the HMT when tested at 100%; 68,966 $\mu g/cm^2$. The values for the other materials are the highest reported concentrations tested that did not give a positive response. All 6 of these nonsensitizers were classified as nonsensitizers in the LLNA (EC_3 greater than 10,000 $\mu g/cm^2$). In the LLNA, hexane, diethylphthalate, and glycerol were tested at 100%; octanoic acid, isopropanol, and acetanisole were tested up to 50%; at all concentrations tested, these materials were uniformly negative.

Discussion

To increase our ability to conduct sound skin sensitization risk assessments, it is imperative that improved methods for quantifying allergen potency and exposure be developed. The critical exposure determination for evaluating skin sensitization risk is dose per unit area of exposed skin.[6,10,36] Use of this parameter allows for comparative assessments amongst different types of skin sensitization tests (including cross-species comparisons). Also critical is the estimation of relative skin sensitization potency. Such information is difficult to obtain from standard guinea pig tests[36-37]; However, the development of the LLNA has provided an opportunity to combine hazard identification with quantitative estimation of sensitizing potency. Specifically, a simple mathematical interpolation of LLNA dose-response data can yield a quantitative estimate of the concentration of test chemical necessary to produce a threshold positive response.[19,20,30] Thus, it is possible to compare allergenic potency determined in the murine LLNA with human values reported in literature from repeat patch testing studies.

The work reported here shows that the potency of 12 out of the 15 chemical allergens were similarly classified by using LLNA EC_3 values and reported human NOELs along with clinical experience (e.g., clinical diagnostic patch testing). A good correlation existed across all potency classifications: strong, moderate, weak, and extremely weak. However, based on our arbitrary classification scheme for the LLNA (Table 3), glutaraldehyde was classified as a strong sensitizer in the LLNA, whereas HRIPT data,[26] together with our expert judgment, classified it as a moderate sensitizer. Propylene glycol, which is classified as an extremely weak human allergen, was classified as a nonsensitizer in the LLNA because no EC_3 value could be determined. Propylene glycol was negative in the LLNA when tested up to 100%. Linalool was classified as an extremely weak human contact allergen based on the available HMT data and our clinical experience. However, linalool was classified as weak in the LLNA based on its EC_3 value being in the range of 1,000 to 10,000 $\mu g/cm^2$ 7,600 $\mu g/cm^2$. For isoeugenol, it is important to point out

that, although the NOEL reported in the literature is 69 $\mu g/cm^2$, the next highest concentration tested that gave a response (LOEL) was 775 $\mu g/cm^2$ (Research Institute for Fragrance Materials/Flavors and Extract Manufacturers, Association Database, Health Data, unpublished data). On the basis of this information, combined with our clinical experience, we classified isoeugenol as a moderate human sensitizer that is consistent with the ranking obtained in the LLNA. Finally, the NOEL used for DNCB was based on a single occluded exposure study,[10] making it difficult to compare directly with the other repeat patch studies. Nevertheless, the compound was classified as strong in the LLNA, which clearly is supported clinically. Overall, the potency ranking of the 15 allergens was very similar in the mouse and human.

For the nonsensitizers, there was strong concordance between the human classification and the quantitative results obtained using the LLNA. Each of the 6 chemicals evaluated and classified as nonsensitizers for humans were classified identically in the LLNA. The chemicals tested at either 50% or 100% gave a negative response in the LLNA (less than 3-fold increase over matched vehicle control). For humans, we have reported the highest concentrations tested, some of which are low compared with those tested in the LLNA. However, clinical experience supports classification of these materials as nonsensitizers. Thus, the potency ranking of these 6 chemicals in the LLNA correlated very well with their apparent inability to cause skin sensitization in humans.

These results strongly support the use of EC_3 values for estimating skin sensitization potency and the use of that information in risk assessment. Importantly, EC_3 values have been shown to be stable with time[31] and reproducible both within[33] and between[32-33] laboratories. However, it is important to recognize that the EC_3 is not an absolute value that can be extrapolated directly to humans, and it might not necessarily relate exactly to the induction threshold in humans. Rather it provides information that enables a comparison of the allergenic potency of one potential sensitizer with that of another and with benchmark chemicals. Importantly, it does this in the context of the vehicle used for the epicutaneous exposure route employed in the LLNA.[38-39] For the chemicals illustrated in this article, the vehicles were not always similar between the presented LLNA and human data. This provides an important advantage to the risk assessor, in that the derived EC_3 values are specific for not only the chemical, but also for the vehicle matrix employed, lending further sophistication to the utility of this information for risk assessment purposes.

In summary, the present investigations reported herein show that the LLNA can be used to provide quantitative estimates of relative skin sensitizing potency (EC_3 values) that correlate closely with what is known of the ability of chemicals to cause skin sensitization in humans. Such information will be of considerable utility in the development of sound risk assessments for skin sensitizing chemicals.

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