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Predictive identification of human skin sensitization thresholds

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For years, methods have been available for the predictive identification of chemicals that possess the intrinsic potential to cause skin sensitization. However, many have proven less suitable for the determination of relative sensitizing potency. In this respect, the local lymph node assay (LLNA) has been shown to have a number of important advantages. Through interpolation of LLNA dose–response data, the concentration of a chemical required to produce a threshold positive response (a 3-fold increase in activity compared with concurrent vehicle controls, the EC3 value) can be measured. The robustness of this parameter has been demonstrated rigorously in terms of inter- and intralaboratory reproducibility. Additionally, the relationship between potency estimates from the LLNA and an appreciation of human potency based on clinical experience has been reported previously. In the present investigations, we have sought to consolidate further our understanding of the association between EC3 values and human skin-sensitization potency by undertaking a thorough and extensive analysis of existing human predictive assays, particularly where dose–response information is available, from historical human repeated insult patch tests (HRIPTs). From these human data, information on the approximate threshold for the induction of skin sensitization in the HRIPT was determined for 26 skin-sensitizing chemicals. These data were then compared with LLNA-derived EC3 values. The results from each assay, expressed as dose per unit area ($\mu g/cm^2$), revealed a clear linear relationship between the 2 values, thereby substantiating further the utility of LLNA EC3 values for prediction of the relative human sensitizing potency of newly identified skin sensitizers.

Key words: human repeated insult patch test; induction thresholds; local lymph node assay; risk assessment; skin sensitization. © Blackwell Munksgaard, 2005.

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For many years, it has been possible to identify chemicals that have the inherent capacity to cause skin sensitization (1–4). Some methods available for this purpose have been shown to be at least 90% accurate (5, 6), with discrepancies often being either of limited importance or due to technical issues for which a resolution has not been critical. However, accurate hazard identification of skin sensitizers represents only the first step in a much more substantial process of safety and risk assessment.

Once a skin-sensitizing chemical has been identified, decisions must be made concerning the extent to which it represents a risk to human health. This requires an appreciation of both the probable conditions and the extent of human exposure to the chemical as well as an understanding of its relative allergenic potency. In addition, where appropriate, warning labelling or consumer information is provided. The considerations that impact on this process depend entirely on an understanding of 2 elements: intrinsic allergenic potency and allergen exposure, the first of which forms the subject of this article. The predictive tests that are used to identify intrinsic hazard (skin sensitization) associated with the substance can also, to varying degrees, provide information on the relative potency of that hazard. This subject has been reviewed recently by various groups (7–10). Of particular note in this respect are the efforts to undertake a simple legislative categorization to discriminate between weaker, moderate and stronger allergens (9, 10). However, potency decisions regarding skin sensitizers are based on predictive test data, most commonly those deriving

from the local lymph node assay (LLNA), and thus, a key question is how accurate are these predictions in terms of the relative potency of skin sensitizers in humans. Initial efforts to demonstrate a relationship between predictive testing in the LLNA and human potency were undertaken in a highly qualitative manner (11). Subsequently, such relationships were refined by defining activity in terms of dose/unit area (μg/cm^2) (12). Most recently, attempts have been made to correlate LLNA data with threshold information derived from published human tests (13, 14). Making such comparisons is fraught with difficulty, however, as the original human data (dating back 40 years ago) was not derived consistently. In the present investigations, we have undertaken a rigorous analysis of the existing human threshold data, rejecting much as being of inadequate quality. The remaining higher quality information has then been compared with the best-quality LLNA data, from which have been derived EC3 values, the quantitative potency index for skin sensitizers (15, 16).

Materials and Methods

Local lymph node assay

LLNA EC3 values, derived by linear interpolation, were taken from the recent LLNA database (17), but wherever possible with the original publication also being cited. Note that in earlier publications, the EC3 value may differ slightly, as the method of derivation was not fixed at that time. In the case of the present work, EC3 values were derived by the approaches detailed in a previous publication (18). These are expressed as dose per surface area (g/cm^2) calculated from concentration applied (%) by using a conversion factor of 250 based on the LLNA OECD protocol (19) using a volume of 25 μl and surface area of 1 cm^2.

The protocol used for the LLNA was as follows: groups of 4 CBA/Ca mice (7–12 weeks of age) were treated topically on the dorsum of both ears with 25 μl of test material or with an equal volume of the vehicle (4 : 1 acetone : olive oil (AOO)) alone. Treatment was performed daily for 3 consecutive days. 5 days following the initiation of exposure, all mice were injected via the tail vein with 250 μl of phosphate-buffered saline (PBS) containing 20 μCi of tritiated thymidine (Amersham International, Amersham, UK). Mice were killed 5 h later and the draining lymph nodes excised and pooled for each experimental group. The lymph node cell (LNC) suspension was washed ×2 in an excess of PBS and then precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18 h. Pellets were resuspended in TCA, and the incorporation of tritiated thymidine was measured by β-scintillation counting. The concentration of the chemical required to produce a stimulation of proliferation of 3 compared with the vehicle-treated control, the EC3 value, was determined to provide a measure of relative skin-sensitizing potential. The EC3 value was calculated by interpolating between 2 points on the SI axis, one immediately above and the other immediately below, the stimulation value of 3. 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 equation:

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

Human repeated insult patch test (HRIPT)

HRIPT data were obtained from the published literature and RIFM-FEMA database (20). For each chemical, a maximal no observed effect level (NOEL) was determined by examination of all sources. In the absence of positive data (where the NOEL was the maximal concentration tested), this has been highlighted. HRIPT data using standard protocols were preferred (21, 22) although with the scarcity of human data this was not always possible. The HRIPT NOEL was expressed as μg/cm^2, using the protocol details given in the study. Patch sizes were as detailed in previous publications (23, 24). In the absence of negative data, a lowest observed effect level (LOEL) expressed as μg/cm^2 was taken provided the percentage of people sensitized was below 8%. Chemicals were excluded where HRIPT protocol details were incomplete or there was only 1 or 2 negative studies conducted with small numbers of people, as low-power HRIPT is unlikely to yield convincing threshold data.

Statistical method

Linear regression analysis of log HRIPT NOELs versus log LLNA EC3 was performed in MICROSOFT EXCEL. 90% confidence and prediction intervals (the latter include the additional variability associated with an individual trial) were produced using Unistat (Unistat® Version 5.5, Unistat Ltd, London, UK). The HRIPT LOELs were plotted on the same graph although these were not included in the regression.

Results

Table 1 contains the complete list of skin-sensitizing chemicals for which we were able to define,

Table 1. Local lymph node assay (LLNA) EC3 and human repeated insult patch test (HRIPT) thresholds in μg/cm^2

Chemical name	CAS number	LLNA EC3	LLNA μg/cm^2	LLNA vehicle	HRIPT threshold μg/cm^2	HRIPT vehicle	References	Graph identifier
1,2-Benzisothiazolin-3-one[a]	2634-33-5	2.3	575	DMF	45	Water	30, 31	A
1,4-Phenylene diamine	106-50-3	0.09	22.5	AOO	10	Petrolatum (pet.)	32, 33	B
2-Methyl-2H-isothiazolone	2682-20-4	1.9	475	AOO	15	Water	18, 34	C
5 Chloro-2-methyl-3(2H)-isothiazolone and 2-methyl-2H-isothiazolone[a]	Mixture	0.009	2.25	DMF	0.83	Water	30, 34	D
Amylcinnamal	122-40-7	10.6	2650	AOO	23 622	DEP	35, 36	E
Benzylidene acetone(4-phenyl-3 buten-2-one)[a]	122-57-6	3.7	925	AOO	1200	Pet.	37, 38	F
Cinnamic alcohol	104-54-1	20.6	5150	AOO	4724	75% DEP, 25% Ethanol	35, 39	G
Cinnamal	104-55-2	3.1	775	AOO	590	Ethanol	40, 41	H
Citral	5392-40-5	13.2	3300	AOO	775	Alcohol SDA39C	42, 43	I
Cyclamen aldehyde	103-95-7	22.3	5575	AOO	4724	Alcohol SDA39C	40, 44	J
Diethyl maleate[a]	141-05-9	5.8	1450	AOO	1600	Pet.	37, 38	K
Ethyl acrylate	140-88-5	28.7	7175	AOO	1600	Pet.	45, 38	L
Eugenol	97-53-0	12.9	3225	AOO	5905	75% DEP, 25% Ethanol	18, 46, 47	M
Formaldehyde	50-00-0	0.65	162.5	Acetone	37	Water	48, 33	N
Geraniol	106-24-1	25.9	6475	75% Ethanol, 25% DEP	3875	75% DEP, 25% Ethanol	49, 50	O
Glutaraldehyde	111-30-8	0.09	22.5	Acetone	100	Pet.	48, 33	P
Hexylcinnamal	101-86-0	11	2750	AOO	23 622	DEP	47, 51, 52	Q
Hydroxycitronellal	107-75-5	33	8250	AOO	2953	75% Ethanol, 25% DEP	40, 53	R
Imidazolidinyl urea	39236-46-9	23.9	5975	DMF	2000	Water	54, 55	S
Isoeugenol	97-54-1	1.7	425	AOO	250	Alcohol SDA39C	46, 56	T
p-tert-Butyl-α-ethyl hydrocinnamal (Lillial)	80-54-6	18.7	4675	AOO	29 525	75% DEP, 25% Ethanol	40, 57	U
Lyral	31906-04-4	17.1	4275	75% Ethanol, 25% DEP	8264	75% Ethanol 25% DEP	23, 58	V
Methyl 2-nonynoate[a]	111-80-8	2.5	625	80% Ethanol	24	75% Ethanol 25% DEP	37, 59	W
Phenyl benzoate	93-99-2	19.6	4900	AOO	9448	DEP	18, 60	Y
Phenylacetaldehyde	122-78-1	3	750	AOO	590	75% DEP, 25% Ethanol	40, 61	Z

AOO, 4 : 1 acetone : olive oil; DEP, diethylphthalate; DMF, dimethylformamide.
[a]EC3 extrapolated as lowest dose gave a stimulation index greater than 3.

with reasonable confidence, a threshold for the induction of skin sensitization in the HRIPT – the NOEL. It also displays the LLNA EC3 values for these 26 substances. HRIPT thresholds ranged from 0.83 to 29 525 μg/cm^2; for these materials, the LLNA EC3 values ranged from 2.25 to 8250 μg/cm^2 (0.009% to 33%). Thus, with this set of data, the range of potencies spanned in both human and murine assays was about 4 orders of magnitude. Allergens, such as the isothiazolinone family, commonly regarded as potent sensitizers in humans, had very low EC3 values. In contrast, much weaker allergens, for example hexylcinnamal, had a very high EC3 value, and corresponding to this they are recognized to be very infrequent human sensitizers, despite considerable exposure in fragrances. Overall, the model explained approximately 70% of the variability in the data.

Figure 1 presents graphically the evidence for a linear relationship between LLNA EC3 values and HRIPT thresholds. As can be seen in Table 1, a large range of solvents have been used across the chemicals and assays used in this analysis. This has also undoubtedly had a detrimental impact on the fit of the model.

4 substances were below the 90% confidence interval, in the order of decreasing LLNA potency: 2-methyl-2H-isothiazolone, 1,2-benzoisothiazolin-3-one, methyl-2-nonynoate and citral. This suggests that the potency has been either underestimated in the LLNA or overestimated in the HRIPT. In addition, a further 4 substances were above the 90% confidence interval, in the order of decreasing LLNA potency: glutaraldehyde, amyl cinnamal, hexylcinnamal and *p*-tert-butyl-α-ethyl hydrocinnamal. For these substances, the LLNA may have overestimated their potency, or the HRIPT has provided an underestimate (Table 2).

Discussion

For many years, the simple paradigm on which skin-sensitization risk assessment was based was a system of classification of skin-sensitizing chemicals into one of a number of categories (weak, moderate, strong, etc.) such as that propounded by Magnusson and Kligman (25) in their 1970 monograph on the guinea-pig maximization test. This type of potency categorization was itself based on the frequency of occurrence of positive skin reactions at challenge, and thus, such categorization was entirely independent of the concentrations of a chemical used either at induction or at challenge. Risk assessors generally had to make a judgement on the probable impact on sensitization induction of the dose levels deployed and factor that in, to form an ultimate view of the risks a particular sensitizing chemical might present in various skin exposure scenarios. Considerable use would be made of benchmarking against other, better known, sensitizing chemicals, such as formaldehyde, particularly where there was knowledge not only of safe uses, but also of awareness, often through clinical data, of use situations associated with the generation of allergic contact dermatitis (ACD). This type

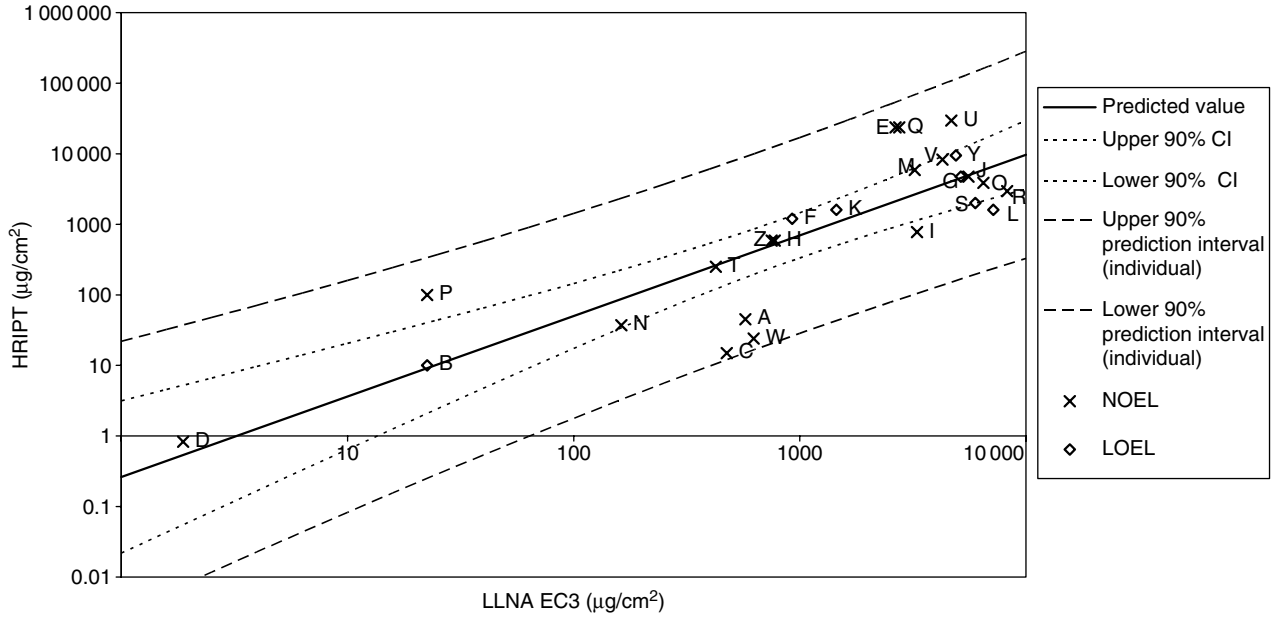

Fig. 1. Linear regression analysis of log human repeated insult patch test (HRIPT) no observed effect levels (NOELs) versus log local lymph node assay (LLNA) EC3. Letters A-Z correspond to identifiers in Table 1. CI, confidence interval.

Table 2. Details of the concentrations, number of people sensitized and evidence of threshold in the human repeated insult patch test (HRIPT)

Chemical name	CAS number	Test concentration (%)	HRIPT result (number of people sensitized/number of people tested)	Evidence of threshold	References
1,2-Benzisothiazolin-3-one	2634-33-5	0.036	0/54	Positive data at higher dose. Note in this article 0.05% likely to be true NOEL	30, 31
1,4-Phenylene diamine	106-50-3	0.01	7/97	LOEL. Lowest dose tested gave positive results	32, 33
2-Methyl-2H-isothiazolone	2682-20-4	0.03	0/98	Dose–response relationship demonstrated in submission	18, 34
5-Chloro-2-methyl-3(2H)-isothiazolone and 2-methyl-2H-isothiazolone	Mixture	0.001	0/602	Dose–response relationship demonstrated in submission	30, 34
Amylcinnamal	122-40-7	20	0/95	Maximum concentration tested	35, 36
Benzylidene acetone(4-phenyl-3-buten-2-one)	122-57-6	3	6/62	LOEL	37, 38
Cinnamic alcohol	104-54-1	4	1/28	LOEL. Mixture of negative and positive data at this concentration, from several studies	35, 39
Cinnamal	104-55-2	0.5	0/38	Dose–response relationship demonstrated in this article, although at 1% subjects reacted at challenge at original site but not at a new site	40, 41
Citral	5392-40-5	1	0/40	Positive HRIPT studies at higher concentrations	42, 43
Cyclamen aldehyde	103-95-7	4	0/64	Maximum concentration tested	40, 44
Diethyl maleate	141-05-9	4	14/187	LOEL	37, 38
Ethyl acrylate	140-88-5	4	4/70	Some NOEL and LOEL at this concentration in the same vehicle. LOEL assumed	45, 38
Eugenol	97-53-0	5	0/108	Maximum concentration tested before threshold. Marzulli and Maibach (38) tested 8% (different vehicles) and determined NOEL and LOEL	18, 46, 47
Formaldehyde	50-00-0	0.037 (tested as formalin)	0/45	Dose–response relationship demonstrated in this article	48, 33
Geraniol	106-24-1	5	0/40	10% (4000 $\mu g/cm^2$) gave a NOEL and LOEL (38)	49, 50
Glutaraldehyde	111-30-8	0.1	0/102	Dose–response relationship demonstrated in this article	48, 33
Hexylcinnamal	101-86-0	20	0/91	Maximum concentration tested	47, 51, 52
Hydroxycitronellal	107-75-5	2.5	0/65	Dose–response relationship demonstrated in this article	40, 53
Imidazolidinyl urea	39236-46-9	2	2/150	LOEL	54, 55
Isoeugenol	97-54-1	0.5	0/53	Positive HRIPT studies at higher concentrations	46, 56
p-tert-Butyl-α-ethyl hydrocinnamal (Lillial)	80-54-6	25	0/106	NOEL/LOEL depending on vehicle; data shown is NOEL	40, 57
Lyral	31906-04-4	15	0/108	Maximum concentration tested	23, 58
Methyl 2-nonynoate	111-80-8	0.02	0/66	Studies at higher concentrations gave positive results	37, 59
Phenyl benzoate	93-99-2	8	1/107	LOEL	18, 60
Phenylacetaldehyde	122-78-1	0.5	0/110	HRIPT studies conducted without anti-oxidants to control peroxide formation were not assessed. Studies with reactions at higher DSA	40, 61

DSA, dose per surface area; HRIPT, human repeated insult patch test; LOEL, lowest observed effect level; NOEL, no observed effect level.

of safety assessment might be referred to as comparative toxicology. Further details of this approach may be found elsewhere, together with discussion of follow-up clinical studies designed as a final premarket check (26).

However, although many skin-sensitizing chemicals are widely and safely used in a wide range of consumer and occupational products, there is no doubt that adequate risk assessments have not always been made by all users of sensitizing chemicals. A most obvious case is perhaps represented by preservatives, where launch onto the market seems often to be followed, to varying extents, by an epidemic of ACD, known as the Dillarstone effect (27). One reason for this is more likely to be the problematic balance between functionality and side-effect.

The data presented in this article aim to do no more than indicate the potential for more soundly based risk assessments that arises from the linear relationship between the thresholds in 2 sensitization assays, the LLNA and the HRIPT. A threshold such as a NOEL represents an important starting point not only for skin sensitization but also for many risk assessments in toxicology. However, many NOELs in toxicology are derived in animal models and thus require interspecies extrapolation. In the case of skin sensitization, the data presented here confirm that the potency profile of mice and man to potential skin sensitizers is broadly equivalent. This knowledge can be harnessed to permit a risk assessment to be made with LLNA data used as a prediction of a NOEL for humans. It is vital to be aware, of course, that this predicted NOEL applies to the HRIPT, not to all potential exposures – the approach to utilizing the NOEL to derive a safe level for a particular type of exposure has been the subject of recent publications (26, 28).

Figure 1 shows the evidence of the association between LLNA EC3 values and HRIPT thresholds. The linear relationship is clear, although not perfect, despite the great care taken in our HRIPT data selection to ensure that only highly defensible data were used. It is our view that a great deal of the unexplained variability still arises from the human data, because it comes from a number of laboratories over a considerable period of time. Here, there is significant contrast with the LLNA data, because EC3 values have been shown to be very robust (29). In summary, LLNA EC3 data permit a prediction of the NOEL in the HRIPT and so provide a solid foundation for the completion of a quantitative risk assessment for skin sensitization.

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