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Skin sensitization in chemical risk assessment: Report of a WHO/IPCS international workshop focusing on dose–response assessment ☆,☆☆

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Abstract

An international workshop was held in 2006 to evaluate experimental techniques for hazard identification and hazard characterization of sensitizing agents in terms of their ability to produce data, including dose–response information, to inform risk assessment. Human testing to identify skin sensitizers is discouraged for ethical reasons. Animal-free alternatives, such as quantitative structure–activity relationships and in vitro testing approaches, have not been sufficiently developed for such application. Guinea pig tests do not generally include dose–response assessment and are therefore not designed for the assessment of potency, defined as the relative ability of a chemical to induce sensitization in a previously naive individual. In contrast, the mouse local lymph node assay does include dose–response assessment and is appropriate for this purpose. Epidemiological evidence can be used only under certain circumstances for the evaluation of the sensitizing potency of chemicals, as it reflects degree of exposure as well as intrinsic potency. Nevertheless, human diagnostic patch test data and quantitative elicitation data have provided very important information in reducing allergic contact dermatitis risk and sensitization in the general population. It is therefore recommended that clinical data, particularly dose–response data derived from sensitized patients, be included in risk assessment.

Keywords: Skin sensitization; Respiratory sensitization; Guinea pig assay; Local lymph node assay; Human patch test; Dose–response assessment; Induction potency; Elicitation potency; QSAR

1. Introduction

In 2004, the International Programme on Chemical Safety (IPCS), a joint venture of the United Nations Environment Programme, the International Labour Organization, and the World Health Organization (WHO), conducted a stocktaking of its project on the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals (IPCS, 2004). An international Harmonization Steering Committee considered proposals

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for new harmonization project activities that had been submitted by risk assessment agencies and individual experts. As a result of the Committee's deliberations, work on skin sensitization was included in the Harmonization Project Workplan for 2005–2006.

IPCS, in conjunction with the German Federal Institute for Risk Assessment, convened an international workshop on skin sensitization in chemical risk assessment in Berlin, Germany, from 17 to 18 October 2006. The workshop focused on skin sensitization arising from exposure to chemicals. It aimed to evaluate experimental techniques for both hazard identification and hazard characterization, with the ultimate goal to evaluate their ability to produce data to inform risk assessment, including dose–response information and information relating to sensitive subpopulations. The workshop focused on whether it is possible to distinguish between chemicals with a high potency to elicit allergic skin reactions and those with a low potency. In addition, emerging approaches, such as structure–activity relationships (SARs), were explored. The meeting also explored whether experimental approaches used in identifying skin sensitization could inform approaches to identify chemicals with the potential for respiratory tract sensitization.

The present article outlines the discussions at the workshop and provides the agreed conclusions and recommendations of the full participants of the workshop. The authors comprise the workshop chair and rapporteur, members of the Planning Group, and the WHO Secretariat. The complete list of participants appears in the Acknowledgments.

2. Background

Potency can be defined as the relative ability of a chemical to induce sensitization, which is determined by the amount of chemical *per unit area* required for the acquisition of skin sensitization in a previously naive individual. Traditional animal test methods used for identification and regulation of skin sensitizers have focused on determining whether or not a substance is a sensitizer. In the traditional guinea pig test methods, the determination is based on results in excess of a predetermined percentage of animals eliciting a response after repeated applications of the substance. In the local lymph node assay (LLNA) in mice, determination that a substance is a sensitizer is based on results exceeding a predetermined ratio of effect in test animals to that in controls. In guinea pig tests submitted for regulatory review, a dose–response assessment is usually not included, although such information is available in some cases. Therefore, guinea pig tests are not designed for looking at potency. With the recent development of the mouse LLNA, the dose at which the stimulating index (SI) of 3 (the threshold for identification of a sensitizer if the test procedure uses radioactive material to identify increased cell proliferation) is exceeded is normally available.

Epidemiological evidence provides information on the prevalence and severity of effects of chemicals in the population owing to sensitization. Clearly, such information is of significance for regulatory purposes. The prevalence of sensitization in the general population is a reflection of the intrinsic potency of the chemical, in addition to the degree of exposure. Moreover, human testing for epidemiological or diagnostic purposes normally measures elicitation responses in subjects who have been previously exposed, not the induction phase of sensitization. Respiratory sensitization is currently classified primarily based on human data because there is no standardized animal model for this endpoint.

3. Workshop questions

The workshop started with a series of lectures (see abstracts in Appendix A) by participants, which formed the basis for the subsequent effort of the workshop: that is, to answer the basic questions as to whether it is possible to distinguish between chemicals with a high potency to elicit allergic skin reactions and those with a low potency, and whether assessment methods for skin sensitization could inform methods for respiratory tract sensitization. These questions were addressed first by discussion in three separate working groups, which considered a series of specific questions (see below). The outcome of these working group discussions was then discussed in a plenary session and is outlined next (see Section 4). The final expert plenary session (see participant details in the Acknowledgments) formulated the agreed conclusions and recommendations of the workshop (see Section 5).

The questions addressed by the three working groups were as follows:

3.1. Group A: Quantification

- What is the most appropriate test for quantification? (validation?)
- Can uncertainty factors be used in sensitization risk assessment? If so, what are they?
- What can we do with existing animal data sets (for existing chemicals) for quantification?
- Can induction predict anything about elicitation in quantitative terms?
- Are there any other related points to be discussed?

3.2. Group B: Human data

- What can we do with existing human data?
- How can quantification be done with human data?
- What is the relative importance of prevalence versus intensity?
- How can human data be validated?
- Are there any other related points for discussion?

3.3. Group C: Hazard identification experimental test methods

- Now that the LLNA is available, should the guinea pig maximization test (GPMT) still be done?
- Are there any circumstances where the GPMT still has a place?
- What are the roles of SARs and in vitro methods?
- What are the roles of variant approaches, such as the cut-down version of the LLNA and non-radioactive approaches?
- Are there any other related points for discussion?

4. Outcome of the working group discussions

4.1. Group A: Quantification

Potency was defined as the relative ability of a chemical to induce sensitization, which is determined by the amount of chemical *per unit area* required for the acquisition of skin sensitization in a previously naive individual (induction phase). Potency does not depend on the assay used because it is an intrinsic property—although different assays may give different results. Any test of skin sensitizing capability that includes dose–response assessment can be used to assess potency. The focus is on animal tests, although human historical data on induction with dose–response can be included. The LLNA incorporates a dose–response assessment and can be used for the categorization of skin sensitization potency. Other animal data sets, including those from the GPMT and the Buehler test, can in principle also be used for this purpose. It is acknowledged that categorization is associated with a degree of uncertainty, which particularly pertains to guinea pig tests, except in the case of substances categorized as extreme sensitizers. Currently, the LLNA is the most appropriate assay for single chemical substances, as it is the only assay that involves dose–response assessment. Expert groups in the European Union (EU) have concluded that in some cases it is possible to categorize a chemical according to its skin sensitization potency (Kimber et al., 2003; Basketter et al., 2005a).

The LLNA has been validated for hazard identification (Kimber et al., 1994, 1995, 1998; Loveless et al., 1996; Gerberick et al., 2000), and a formal validation for regulatory purposes was performed by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 1999; Dean et al., 2001; Sailstad et al., 2001). In addition, there are considerable data in the open literature to indicate its suitability for measurement of potency and the validity of such potency measurements (Loveless et al., 1996; Hilton et al., 1998; Basketter et al., 1999, 2000, 2005b; van Och et al., 2000; de Jong et al., 2002; Schneider and Akkan, 2004). More limited data sets have been published for the guinea pig tests (Andersen et al., 1995). Given this history, modifications of these procedures should not require the same degree of validation as the original methods. However, an abbreviated approach may be appropriate to assess the validity of potency assessment based on the LLNA and its appropriateness for predicting potency in humans. The suitability of testing of mixtures and preparations, including assessment of potency, is not established for any sensitization assay.

The working group concluded that adjustment factors can be used in sensitization risk assessment. In general toxicology, adjustment factors are applied to extrapolate from experimental data in animals to the human population. These adjustment factors account for interspecies differences (Travis and White, 1988; IPCS, 2005) and human (interindividual) variability (Renwick and Lazarus, 1998; Burin and Saunders, 1999; Aldridge et al., 2003; IPCS, 2005). In dermal sensitization risk assessments, it is equally necessary to extrapolate from the experimental exposure (defined and controlled exposure conditions) to real-life exposure (variable exposure controlled by the individual). One detailed proposal for how this could be achieved is by the application of a sensitization assessment factor (SAF). The SAF takes into account three parameters—interindividual variability (the same as in general toxicology), vehicle/product matrix effects, and use considerations (specific for dermal sensitization, including site of contact, dermal integrity, and occlusion) (QRA Expert Group, 2006). The LLNA EC3 (the effective concentration inducing an SI of 3) value has recently been demonstrated to correlate with non-sensitizing levels (no-observed-effect levels, or NOELs) in historical human repeat insult patch tests (HRIPT) (Gerberick et al., 2001; Griem et al., 2003; Basketter et al., 2005b) and therefore provides a route to the predictive identification of HRIPT NOELs without the necessity for human testing.

Although there are many mechanistic similarities between induction and elicitation, in reality it is not normally possible to predict anything about elicitation from an appreciation of induction potency. The general paucity of information in this area caused an EU expert group to conclude that ''variation in elicitation thresholds between individuals is very large and depends on numerous factors of which the sensitizing potency of the substance is only one. Other factors affecting elicitation include the duration, extent and site of exposure, status of the skin, and degree of specific sensitization. For this reason, the expert group considered that it would be inappropriate to define elicitation thresholds as a function of skin sensitizing potency'' (Basketter et al., 2005a).

Using the currently available tests for determining skin sensitization potency, no information will be yielded to predict respiratory sensitization potency.

4.2. Group B: Human data

Several types of human data are available for assessing risk of allergic contact dermatitis (ACD), including

epidemiology, case studies, dose–response elicitation studies in sensitized individuals, and induction studies in human volunteers. Because of ethical considerations, induction studies are limited to historical data (SCCNFP, 2000; Menné and Wahlberg, 2002). Epidemiological investigations can provide hazard identification and exposure assessment information. Data include studies of the general population, occupational or non-occupational cohorts, or dermatitis patients and may consist of patch testing and/or questionnaire/survey data. Further dose–response elicitation studies in individuals diagnosed with contact allergy provide quantitative information. To date, human diagnostic patch test data and quantitative elicitation data (e.g., patch test, repeat open application insult test [ROAT], use test) have provided very important information in reducing risk to ACD in sensitized patients as well as in reducing sensitization in the general population. The prevalence of ACD to certain chemicals is increasing, possibly due to increased exposure, whereas it is decreasing for other chemicals owing to elimination or decreased utilization of the chemicals in products based on clinical testing results in dermatitis patients (Jensen et al., 2002; Wilkinson et al., 2002; Schnuch and Uter, 2003). The severity of the clinical disease depends on the degree of sensitivity of the individual, the type of exposure (e.g., time, intensity, frequency), and the nature of the allergen, all of which may affect the outcome of a study.

It was concluded that when positive clinical data from sensitized individuals are available, those data should be given priority for use in hazard assessment over other predictive data (e.g., animal data or human induction data), because they were deemed to be the most sensitive and relevant for prevention of clinical disease. However, negative clinical data should not normally be used to override positive animal data. It was recognized that for new chemicals or existing chemicals with unknown effects, human data may not be available. In these cases, hazard identification and quantification must rely primarily on animal data, although all available information should be taken into consideration as part of the weight of evidence. In this context, it is also important to demonstrate, through monitoring systems, that preventive actions taken based on animal data have an impact on the prevalence of disease in the population.

Because it is desirable to use human dose–response data for quantitative risk assessment, it is recommended that dose–response curves from patch testing and/or ROAT be derived in individuals diagnosed with contact allergy in order to establish a threshold, which can be used as a point of departure for risk assessment. There is a need for a standardized system for classifying and determining limits according to potency. It is recognized that elicitation responses reflect both potency and exposure and that potency cannot be directly derived from human elicitation data. However, a low elicitation threshold is suggestive of high potency.

The nature of clinical studies makes validation of human data a challenge. Reproducibility of results among multiple studies by the same and different investigators and comparability between data from use tests and diagnostic patch tests are possible means of validation. To facilitate comparisons between studies using the patch test and other diagnostic tests, these tests should be performed using standard clinical guidelines (Frosch et al., 2006). Patch test data together with documented exposure imply causality. It is recognized that differences in results among different studies may be related to intrinsic factors that play a role in susceptibility. Differences in susceptibility are important considerations in risk assessment; at this time, however, there is insufficient information on this topic to apply in risk assessment.

The working group agreed that the ability to use human data would be improved by generating prevalence data in the general population and surveillance data on the impact of measures taken to reduce ACD. More work is needed to determine what uncertainty factors should be applied. In this regard, a better understanding of the role of genetics in susceptibility and the degree of variability in responses would be useful. Issues related to exposure assessment also need to be addressed, such as comparison of occluded versus non-occluded exposures, single versus repeated exposures, and methods for measuring and modelling skin exposure.

4.3. Group C: Hazard identification experimental test methods

The working group agreed that the LLNA is the preferred test method for assessing the skin sensitization capability of chemicals in view of animal welfare considerations. The LLNA has been validated for the purpose of hazard identification. It is noted that the LLNA is the test of choice in the forthcoming EU REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) programme. However, there is still an issue of irritant responses in the LLNA, which needs to be further addressed. In addition, it was agreed that there is still a need for guinea pig tests. If the LLNA cannot be used—for example, for the testing of aqueous solutions, extracts, fabrics, mixtures, and preparations—then the conduct of guinea pig tests may be appropriate. Although guinea pig tests have never formally been validated for the purpose of sensitization testing, they have been used historically and have been shown to be fit for purpose. When conducting guinea pig assays, the Buehler assay has preference over the GPMT from an animal welfare point of view. However, the GPMT is generally considered to be more sensitive than the Buehler assay, for which reason some regulatory authorities prefer the GPMT for hazard identification. Further development and adaptation of the LLNA are therefore needed with a view to testing of aqueous solutions and testing of

preparations or complex mixtures. Such development may include in-ear dosing to circumvent issues with aqueous solutions.

The reduced version of the LLNA (Kimber et al., 2006) may be of great value, especially where the screening of large numbers of compounds is necessary. However, it is recognized that the future development of classification categorization may require a full LLNA to be conducted for potency identification for those chemicals positively identified.

Some countries have restrictions with regard to the use of radioactivity for the LLNA. Ex vivo in vitro labelling may be used in order to reduce radioactive waste, and there is evidence of the validity of this approach. The further development of non-radioactive approaches is necessary.

(Quantitative)SARs or (Q)SARs, based upon existing knowledge, may be useful as part of a weight-of-evidence approach for identification of the sensitizing capacity of chemicals. There are a number of local (Q)SARs that can be used for a limited range of chemicals, where ''local'' implies a focused model typically characterized by a chemical class or single chemical mechanism of action. However, there are currently insufficient local (Q)SARs to cover the whole chemical universe. Currently, the feeling is that (Q)SARs and expert systems may be used as part of a weight-of-evidence approach but should not be used as a stand-alone method to identify hazard. There is therefore a need to clearly establish the applicability domain of each model, to do more work to characterize chemical reactivity, to develop the predictive capacity of these models of skin metabolism, and to increase the ability for predicting negatives.

With regard to in vitro test systems for sensitizing capacity, there are currently a number of in vitro methods at various stages of development. None of these has been validated for hazard identification purposes. Some of these systems may be useful in a weight-of-evidence approach or as preliminary screens. Perhaps the most promising assay is the direct peptide assay. In general, for cell-based assays, further development is needed. New opportunities, such as the development of three-dimensional skin constructs that incorporate immunocompetent cells and allow for topical application of test articles, may offer new avenues of testing and should be further explored. A combination of in vitro assays, perhaps as part of a tiered approach, may be required for the prediction of skin sensitization hazard.

Most, if not all, respiratory sensitizers so far have tested positive in the LLNA. There are indications that cytokines produced by the draining lymph node cells after skin exposure might identify respiratory sensitizers (Dearman et al., 1996; Vandebriel et al., 2000). Further research is needed to establish whether and how the LLNA can be used to identify respiratory sensitizers.

5. General conclusions and recommendations of the workshop[1]

5.1. Conclusions

The relative ability of a chemical to induce sensitization is an intrinsic property of the chemical and is determined by the amount of chemical per unit area required for the acquisition of skin sensitization in a previously naive individual. The LLNA is the preferred test method for assessing the skin sensitization capability of chemicals in view of animal welfare considerations. It has been validated for the purpose of hazard identification. At present, however, there is still a need for guinea pig tests. Guinea pig tests may still have a place in the testing of aqueous solutions, extracts, fabrics, mixtures, and preparations. When conducting guinea pig assays, the Buehler assay is preferred over the GPMT from an animal welfare point of view. However, the GPMT is generally considered to be more sensitive than the Buehler assay, for which reason some regulatory authorities prefer the GPMT.

(Q)SARs and expert systems for identification of sensitizing capacity have not been validated to date, but may be used as part of a weight-of-evidence approach for identifying the sensitizing capacity of chemicals. There are certain local (Q)SARs that can be used for a small range of chemicals. However, these are currently insufficient to cover the full range of chemicals.

No in vitro assay systems for the identification of sensitizing capacity have been validated to date. Some of these systems may be useful in a weight-of-evidence approach or as a preliminary screen.

Any test of skin sensitizing capability that includes dose–response assessment can be used to assess potency. Currently, the LLNA is the most appropriate assay for single chemical substances, as it is the only test for which guidelines indicate the inclusion of dose–response assessment. Guinea pig data may also be used to categorize a chemical according to its skin sensitizing potency. It is acknowledged that categorization of skin sensitizing potency is associated with a degree of uncertainty. Neither the approach using the LLNA nor the approach using guinea pig data has been validated for the purpose of assessment of potency.

Elicitation responses depend on several factors, among which are potency of the allergen and exposure conditions. Even though potency cannot be directly derived from human elicitation data, a low elicitation threshold is suggestive of a high potency. Where possible, attempts should be made to use clinical data for quantitative risk assessment.

The suitability of test methods for mixtures and preparations, including assessment of skin sensitization induction potency, is not established for any sensitization assay.

[1] The conclusions and recommendations were agreed by full participants of the workshop.

Elicitation thresholds cannot be determined on the basis of skin sensitizing potency.

Although respiratory allergens tested so far were positive in current tests evaluating skin sensitization potential, skin sensitization potency data available from current test methods do not predict respiratory sensitization potency.

5.2. Recommendations

1. There is a need for a standardized system of classifying and determining limits according to potency.
2. The use of the LLNA for potency categorization of induction of skin sensitization needs to be validated. An abbreviated test validation approach may be appropriate to assess the validity of potency assessment based on the LLNA and its appropriateness for predicting sensitizing induction potency in humans.
3. It is recommended that dose–response curves be derived from patch testing and/or open testing in individuals diagnosed with contact allergy, thereby establishing a threshold that can be used to derive a point of departure for risk assessment.
4. Existing human data on variability in individual thresholds should be evaluated to derive adjustment factors for risk assessment.
5. It is recommended that further studies be carried out regarding ranking of chemicals according to their potency to elicit allergic responses in individuals diagnosed with contact allergy.
6. Comparison of information on responses after occluded versus non-occluded exposures and after single versus repeated exposures should be done to inform adjustment factors for risk assessment that may account for specific exposure conditions.
7. Methodology to assess skin penetration, deposition, and metabolism needs to be further advanced.
8. The LLNA needs to be further developed with a view to testing of aqueous solutions, preparations, and complex mixtures.
9. The effects of irritant activity in the LLNA should be further explored.
10. It is recommended that non-radioactive forms of the LLNA, or LLNA-type assays that use reduced amounts of radioactivity, receive more attention.
11. It is recommended that QSAR models be further developed and that the applicability domain of each model be established.
12. Approaches to evaluate respiratory sensitization induction potency need to be developed.

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Appendix A. Abstracts

The use of human data when conducting dermal sensitization quantitative risk assessments for fragrance ingredients

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1. Introduction

Historical human data from either human repeated insult patch tests (HRIPTs) or human maximization tests (HMTs) are available for raw materials found in consumer products and for a variety of those products. This is certainly true for fragrance ingredients. These data add an important aspect to the overall evaluation, based on a weight-of-evidence approach, of dermal sensitization for a fragrance ingredient when conducting a quantitative risk assessment (QRA). In fact, the HRIPT is currently the primary way of confirming in humans a predicted dermal sensitization no-observed-effect level (NOEL) from animal testing.

2. Main points

A human sensitization test is not used to determine hazard. The test is not used as a predictive method, nor is it used on substances with unknown dermal sensitization potential. It is a test used to confirm the lack of dermal sensitization at an exposure level that was identified as a NOEL in an animal model or derived as a likely NOEL from quantitative structure–activity relationships (QSARs).

Human patch testing methodology has evolved over more than 50 years. In every method, a number of induction exposures are followed by a rest period and then a challenge exposure, but variations exist as to patch type, number of subjects, skin site, number of induction patches, patch application time, duration, and rest period prior to challenge. In all of the methodologies, enhancement of the skin response after challenge over that seen during early induction exposures has been the criterion by which induction of contact allergy is measured. Test volunteers are typically healthy adults who are enrolled without restriction as to sex or ethnicity. The test most typically conducted is the HRIPT.

In HRIPTs, the size of the test population is important with regard to interpretation of findings. The sample size of test subjects must be sufficiently large so that results are valid for the population at large, yet small enough to be logistically feasible to conduct the study. Henderson and Riley (1945) investigated statistical calculations of patch tests adapted for the detection and evaluation of chemicals for dermal sensitization. If no reactions are observed in a group of 100 test subjects, the rate of positive reactions in a larger population is not likely to exceed 2.9%, based on a confidence level of 95%, *under identical conditions.* The likely maximum rate of 2.9% positive reactions is often misinterpreted to mean that there would be an expected rate of 2.9% in the marketplace general population. The test conditions in the HRIPT are not identical to real-life scenarios. To increase the sensitivity of the test while using such numbers of subjects, if appropriate, one generally tests a higher concentration of test material and possibly more exaggerated exposure conditions than would actually be encountered in intended and foreseeable use situations among the general population. Other factors that further increase the sensitivity and reliability of the test, in some HRIPT protocols, are exaggeration through possible minor skin irritation of a test material, use of occluded patches, and vehicle effects from the test conditions (Basketter et al., 2006).

The induction of human dermal sensitization from the HRIPT is rare. Hall (2006) estimated the rate of dermal sensitization induction to be 0.09% of volunteers in tests on cosmetic products. In addition, Hall (2006) noted that there has been no evidence of adverse sequelae from these tests.

With implementation of the QRA approach, the International Fragrance Association (IFRA) and the Research Institute for Fragrance Materials (RIFM) are recommending the use of the RIFM standard HRIPT protocol for generation of confirmatory human data for use in QRA (QRA Expert Group, 2006). Details of this standard HRIPT protocol are available from RIFM.

RIFM has a historical database that contains more than 1000 HRIPTs and more than 1200 HMTs conducted on individual fragrance ingredients. In addition, there are more than 20 years of experience in using the HRIPT as a confirmatory assay by RIFM. This accounts for more than 200 HRIPTs on file that have been conducted by RIFM using the same (RIFM standard) protocol. In addition, the RIFM database contains a significant and

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Table 1
Comparison of murine local lymph node assay EC3 values with the NOELs from confirmatory human sensitization tests

Fragrance ingredient	CAS No.	LLNA weighted mean EC3 values (μg/cm^2) (No. of studies)	Human data			Potency classification[b]
			NOEL HRIPT (induction) (μg/cm^2)	NOEL HMT (induction) (μg/cm^2)	LOEL[a] (induction) (μg/cm^2)	
Very good correlation						
α-Amylcinnamyl alcohol	101-85-9	>6250 (1)[c]	3543[d]	NA	NA	Weak
Anisyl alcohol	105-13-5	1475 (1)[c]	NA	3448[d]	NA	Weak
Benzyl benzoate	120-51-4	>12,500 (1)[c]	59,050[d]	20,690[d]	NA	Extremely weak
Benzyl cinnamate	103-41-3	4600 (1)[c]	4720[d]	5517[d]	NA	Weak
para-tert-Butyl-α-Methylhydrocinnamic aldehyde (BMHCA)	80-54-6	2372 (6)	4125	NA	29,528	Weak
Cinnamyl alcohol	104-54-1	5250 (1)[c]	3000	2759	4724	Weak
Cinnamaldehyde	104-55-2	262 (23)	591	NA	775	Moderate
Cinnamyl nitrile	1885-38-7	>2500 (1)[c]	1476	NA	NA	Weak
Citral	5392-40-5	1414 (11)	1400	NA	3876	Weak
DL-Citronellol	106-22-9	10,875 (1)[c]	29,528[d]	4138	NA	Extremely weak
Coumarin	91-64-5	>6250 (1)[c]	3543	5517	8858	Weak
Eugenol	97-53-0	2703 (6)	5906	NA	NA	Weak
Farnesol	4602-84-0	1200 (2)	2755	NA	6897[e]	Weak
Geraniol	106-24-1	3525 (5)	11,811	NA	NA	Weak
Hydroxycitronellal	107-75-5	5612 (9)	5000	NA	5906	Weak
Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HMPCC)	31906-04-4	4275 (1)[c]	4000	NA	NA	Weak
Isocyclogeraniol	68527-77-5	>6250 (1)[c]	3898	NA	7752	Weak
Isoeugenol[f]	97-54-1	498 (18)	250	NA	775	Moderate
D-Limonene[g]	5989-27-5	10,075 (5)	10,000[d]	5517[d]	NA	Weak
Linalool[g]	78-70-6	12,650 (2)	15,000[d]	13,793[d]	NA	Extremely weak
Methyl 2-octynoate (methyl heptine carbonate)	111-12-6	<125 (1)[c]	118	NA	194	Strong
Methyl 2-nonynoate (methyl octine carbonate)	111-80-8	<1250; estimated 625 (1)[c]	24	NA	118	Strong
Phenylacetaldehyde	122-78-1	962 (2)	591	NA	1181	Moderate
Oakmoss[h]	90028-68-5	2476 (4)	700	NA	NA	Moderate
Treemoss[i]	90028-67-4	2163 (2)	700	NA	NA	Moderate
Less predictive correlation						
α-Amylcinnamaldehyde	122-40-7	2942 (3)	23,622[d]	NA	NA	Extremely weak
Benzyl alcohol	100-51-6	>12,500 (1)[c]	5906	6897	8858	Weak
Benzyl salicylate	118-58-1	725 (1)[c]	17,717[d]	20,690[d]	NA	Weak
trans-2-Hexenal	6728-26-3	1012 (2)	24	NA	236	Strong
α-Hexyl-cinnamaldehyde	101-86-0	2372 (>5)	23,622[d]	NA	NA	Weak
α-*iso*-Methylionone	127-51-5	5450 (1)[c]	70,866[d]	NA	NA	Weak

All data in this table are available from RIFM and are listed in the RIFM database.

CAS, Chemical Abstracts Service; HMT, human maximization test; HRIPT, human repeated insult patch test; LLNA, local lymph node assay; LOEL, lowest-observed-effect level; NA, not available; NOEL, no-observed-effect level.

[a] Data derived from HRIPT or HMT.

[b] Gerberick et al. (2001a).

[c] EC3 value from one LLNA, not the mean.

[d] MT-NOEL, maximum tested no-observed-effect level. No sensitization was observed in human studies. Doses reported reflect the highest concentration tested, not necessarily the highest achievable NOEL.

[e] LOEL from HMT, not HRIPT.

[f] Isoeugenol potency classification is listed as "moderate" because the LOEL is 775 μg/cm^2. A moderate classification is consistent with isoeugenol's potency in animal tests.

[g] D-Limonene and linalool are not contact allergens, but some hydroperoxides formed by autoxidation are known to be dermal sensitizers. In addition, D-limonene and linalool are known human irritants. The irritancy profile of D-limonene and linalool is being further investigated by RIFM.

[h] Oakmoss: data on untreated qualities; new qualities of oakmoss, which contain significantly lower levels of atranol and chloratranol, are under test.

[i] Treemoss: data on untreated qualities; new qualities of treemoss, which contain significantly lower levels of atranol and chloratranol, are under test.

increasing number of murine local lymph node assays (LLNAs) that can be used in combination with the confirmatory human dermal sensitization data.

The EC3 value determined from the LLNA is the concentration required to induce a threshold positive response (stimulation index equal to 3). The most robust and convenient method for the routine calculation of EC3 values is to derive it by linear interpolation from the dose–response data (Basketter et al., 1999). The EC3 value has recently been demonstrated to correlate closely with the NOEL from human sensitization tests designed to confirm lack of induction (Basketter et al., 2000, 2005; Gerberick et al., 2001a,b, 2004; Griem et al., 2003; Schneider and Akkan, 2004).

A detailed analysis of the dermal sensitization data for 31 fragrance ingredients that have exhibited dermal sensitization potential revealed that for the majority of the materials, there is a very good correlation between the EC3 or predicted NOEL from the LLNA and the NOEL in confirmatory human tests. Table 1 provides details of the data on these fragrance ingredients. The data show that for 25 of 31 of the fragrance ingredients reviewed, there is a very good correlation between the EC3 value from the LLNA and the NOEL in confirmatory human dermal sensitization tests. For the remaining six materials, the correlation is less predictive. Of these six materials, the data for four reveal that the LLNA EC3 value is much lower than the maximum tested NOEL in humans (no sensitization was observed in the confirmatory human studies; the dose reported reflects the highest concentration tested, not the highest achievable NOEL). The absence of significant clinically relevant positive reactions in dermatology clinics provides support for these data. However, for two materials (benzyl alcohol and *trans*-2-hexenal), the data show that the EC3 value overestimates the NOEL in confirmatory human tests. These data illustrate the importance of conducting a confirmatory human sensitization test.

The reason for this lack of correlation is not currently fully understood. It may be due to the amount of material that is absorbed and/or differences in the metabolic capabilities of mouse and human skin.

3. Conclusions and future directions

- A human dermal sensitization test is not used to determine hazard; rather, it is a test used to confirm the lack of sensitization at an exposure level that was identified as a NOEL in an animal model or derived as a likely NOEL from QSARs.
- The induction of dermal sensitization from confirmatory human tests is rare, because the assay is used to confirm a NOEL.
- The confirmatory HRIPT methodology is robust in design in terms of number of individuals, exposure conditions, and evaluation parameters. The test conditions in the HRIPT are exaggerated compared with real-life scenarios and are relevant to the generation of data that are very important to the application of a QRA approach.
- The EC3 value has recently been demonstrated to correlate closely with the NOEL from confirmatory human sensitization tests designed to confirm lack of induction.
- A detailed analysis of the dermal sensitization data in the RIFM database for 31 fragrance ingredients that have exhibited dermal sensitization potential revealed that for the majority of these fragrance ingredients, there is a very good correlation between the predicted NOEL from the murine LLNA and the NOEL in confirmatory human tests.

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Application of the local lymph node assay (LLNA) for respiratory sensitizers

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1. Background

Allergy is a two-step immunological process in which sensitization is the first step. The sensitization phase is a symptomless phase that can be followed by an elicitation phase upon subsequent encounter with the chemical in which the adverse health effects develop. Immune responses may be polarized towards either Thelper1 (Th1)- or Thelper2 (Th2)-mediated allergic reactions. Allergic contact dermatitis (mainly Th1) is the most common allergic disorder in the skin. Asthma and allergic rhinitis (mainly Th2) are most frequently encountered in the respiratory tract, asthma being so prominent that respiratory allergy has become almost synonymous with asthma. Thus, based on human evidence, the skin appears more prone to Th1 allergic reactions, whereas the respiratory tract appears more prone to Th2 allergic disorders. This concept is in use to test chemicals on their potential to cause skin and/or respiratory allergy, although it is recognized that it is an oversimplification. Skin allergy may also express itself as an immediate type (Th2-mediated) hypersensitivity reaction (e.g., atopic dermatitis), and respiratory allergy also includes allergic alveolitis (hypersensitivity pneumonitis; mainly Th1).

The local lymph node assay (LLNA) is used to test the potential of low molecular weight compounds to induce skin sensitization. It measures proliferation of lymphocytes in lymph nodes draining the route of application. These are the auricular lymph nodes, because animals receive the test material on the ears. The LLNA would be suitable to test not only compounds that are of a Th1 type, but also those of a Th2 type, if it is assumed that lymphocyte proliferation is induced in draining lymph nodes regardless of Th1- or Th2-type allergy. Indeed, there is evidence that chemical respiratory (Th2) allergens will also elicit positive responses in this assay (Kimber, 1995; van Och et al., 2000). However, it should be kept in mind that all known respiratory allergens are classified as such based on human evidence and may therefore be considered strong allergens.

To test the hypothesis that sensitizers stimulate draining lymph nodes following inhalation exposure, in analogy to dermal exposure, we developed a respiratory LLNA. In the respiratory LLNA, we tested whether potential and potency via the dermal route are comparable with those via the inhalation route.

2. Main points

1. Because allergic reactions in the skin have a tendency to develop as Th1-type allergic reactions, would the dermal LLNA be sensitive enough to detect the sensitizing potential of weakly or moderately potent Th2 allergens?
2. Could the skin application route result in an underestimation of the sensitizing potency of respiratory allergens because of the larger mechanical barrier in the skin compared with the airways?
3. Can compounds that are gaseous or have a very high volatility be tested in an appropriate way in the dermal LLNA?

2.1. Dermal LLNA and hazard identification

In the dermal LLNA, a stimulation index (SI) of at least 3—that is, an at least three times higher proliferation than that of the controls—designates a compound a sensitizer. The corresponding EC3 value, the effective concentration inducing an SI of 3, is used to compare the sensitizing potency of compounds.

2.2. Respiratory LLNA

Trimellitic anhydride (TMA; 30 mg/m^3), phthalic anhydride (PA; 15 mg/m^3), hexamethylene diisocyanate (HDI; 15 mg/m^3), and toluene diisocyanate (TDI; 7.5 mg/m^3) were used as model respiratory allergens; dinitrochlorobenzene (DNCB; 30 mg/m^3) and oxazolone (OXA; 30 mg/m^3) were used as model skin allergens. Groups of six male BALB/c mice were exposed nose-only for 45, 90, 180, or 360 min/day on three consecutive days. The dermal route (ear application; $n = 3$) was used as a positive control. Negative controls ($n = 12$) were exposed by ear application of the vehicle (acetone/olive oil, 4:1) and by inhalation of the vehicle (acetone) for 360 min/day for 3 days. The animals were necropsied 3 days after the last exposure,

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and the local lymph nodes were excised. Harvested lymph node cells were cultured in vitro with [^{3}H]thymidine to determine proliferation. In addition, production of the cytokines interferon-γ (IFN-γ) and interleukin-4 (IL-4) was measured by the enzyme-linked immunosorbent assay (ELISA) after co-culture with concanavalin A. In the inhalation groups, lymph nodes draining various parts of the respiratory tract, including nasal passages and nasopharynx, larynx/trachea, and trachea/bronchi/bronchioli, were grossly examined, because the impact of compounds in the respiratory tract and (thus) their exact draining pattern are often not fully known. The auricular lymph nodes were sampled in the positive control group.

3. Results

All allergens induced grossly observed enlargement of and proliferation in the lymph nodes draining the upper respiratory tract (mandibular lymph nodes). This is not surprising, as the impaction of compounds is usually high in the upper respiratory tract. Increased IFN-γ was found with all allergens tested (DNCB, OXA, TMA, and HDI), whereas increased IL-4 was found with the typical respiratory allergens TMA and HDI only. With regard to potency ranking, the typical contact allergens DNCB and OXA were at least of comparable potency to TMA in the respiratory LLNA.

4. Discussion and conclusions

- The dermal LLNA is a promising tool to detect Th2 respiratory allergens, but the dermal LLNA may not be suitable to establish correct ranking of respiratory sensitizers.
- The use of the dermal LLNA may be compromised by the physicochemical characteristics of a compound (gaseous or very high volatility).
- In analogy to the dermal LLNA, the present results in the respiratory LLNA suggest that strong contact allergens such as DNCB and OXA can also act as potent sensitizers by inhalation, provided that such compounds are inhaled.
- With regard to cytokine production, IL-4, but not IFN-γ, seems to be able to discriminate between typical respiratory and contact allergens.

5. Future directions

- The dermal LLNA is a promising tool to detect respiratory allergens. However, it remains to be established whether the dermal LLNA is sensitive enough to detect weak or moderate respiratory sensitizers. Therefore, a few moderate respiratory allergens and strong respiratory irritants need to be tested in the respiratory LLNA and compared with the dermal LLNA.
- It still has to be investigated whether potency is best characterized by dose–response data or EC3 values.

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Information derived from sensitization test methods: Test sensitivity, false positives, and false negatives

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1. Introduction

Any toxicological test must fulfil some basic requirements to be of practical value in the protection of human health. It must be relevant to the endpoint of concern (in this case, skin sensitization) and reliable—that is, reproducibly able to give the correct prediction when the test is repeated, either at the same laboratory or in different locations. The process by which this requirement is demonstrated for new/in vitro tests is called ''validation'', and this activity has been very clearly prescribed (Zeiger and Stokes, 1998; Worth and Balls, 2002).

2. Main points

Tests for the prospective identification of chemicals that possess the ability to cause significant skin sensitization have been available for over half a century (reviewed in Andersen and Maibach, 1985; Botham et al., 1991; Basketter, 1994; Steiling et al., 2001). These tests have normally used the guinea pig as the species of choice, following on from studies conducted early in the last century (e.g., Landsteiner and Jacobs, 1936). Of the many methods described, none of which was formally validated, ultimately only the guinea pig maximization test (GPMT) of Magnusson and Kligman (1970) and the occluded patch test of Buehler (1965) have continued to be accepted. Relatively recently, an alternative model using the mouse, the local lymph node assay (LLNA), has gained widespread acceptance following formal validation and acceptance in the USA and in Europe (ICCVAM, 1999; Balls and Hellsten, 2000).

2.1. Test sensitivity

Following the earliest development of guinea pig methods that were of limited sensitivity, first Buehler (1965, 1985) and then Magnusson and Kligman (1970) proposed protocols designed to offer sufficient sensitivity to identify weaker as well as strong skin sensitizers. All of these assays employed dermal routes of exposure and occlusion during the induction phase, followed by a topical application challenge to demonstrate whether any degree of skin sensitization had been induced. The occluded patch test reported by Buehler (1965, 1985), however, was originally not sufficiently well described and proved very susceptible to technical variations that could severely compromise the assay. This in part prompted Magnusson and Kligman (1970) to report very thoroughly the work they had done to establish the GPMT. However, in these laudable efforts to enhance test sensitivity, little consideration was given to the matter of specificity—that is, the risk of increasing the number of false positives at the expense of reducing the number of false negatives (Kligman and Basketter, 1995). Furthermore, much of this occurred at a time when there was no requirement for validation, independent or otherwise, of any protocol.

In recent years, the LLNA has been widely adopted (Gerberick et al., 2000). In this assay, sensitizing activity is detected as a function of the cell proliferation triggered in lymph nodes draining the site of epicutaneous application. This assay was subject to the full rigour of independent validation, with 200+ chemicals being used to demonstrate its sensitivity and specificity (ICCVAM, 1999; Gerberick et al., 2000).

2.2. False positives

All predictive toxicology tests have limitations. The incorrect identification of a chemical with very limited or no sensitizing activity (for humans) as positive is bound to occur. This might occur for a variety of reasons, including interspecies differences or the fact that the endpoint of the predictive test is not a direct mechanistic correlate of the true human process. For the guinea pig tests of lesser sensitivity, false-positive results were rarely reported as a practical problem. However, for the highly sensitive GPMT, false positives were considered more likely, eventually with Kligman himself describing the problem and potential solutions (Kligman and Basketter, 1995). One of the first described GPMT false-positive results reported was for sulfanilic acid (Basketter et al., 1992). This chemical was correctly identified as non-sensitizing in the LLNA, but this assay reported the classic irritant sodium lauryl sulfate (SLS) as a false positive (Basketter et al., 1998). The characterization of false positivity in each of these cases rested heavily on human experience, demonstrating that despite extensive skin exposure, neither of these

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chemicals had been found to sensitize humans. Of potential importance in this respect may be the requirement within the GPMT for intradermal exposure to the test chemical in combination with a powerful adjuvant.

2.3. False negatives

In this situation, substances that in reality should be recorded as skin sensitizers fail to cause a (sufficient) response in the test system. To a great extent, this was the driving force behind the development of the GPMT. Thus, false negatives may be less common in this method, but they still exist, an obvious example being the paraben family of preservatives, which are well-described human sensitizers; a key question, however, is whether they are really of sufficient sensitizing power to merit formal classification (Basketter et al., 2006). Paraben is also negative in other predictive assays. Often, apparent false negatives may be the result of poor quality test conduct, for example, with methyldibromoglutaronitrile (Basketter et al., 2006). This preservative caused an epidemic of allergic contact dermatitis and was subsequently shown to be a strong sensitizer in the LLNA. Such divergent results may arise simply as a consequence of the many technical challenges of conduct of the guinea pig test methods, where factors such as the efficacy of fur removal and quality of patch occlusion can have a dramatic impact on test outcome (Basketter, 1994).

3. Conclusions and future directions

If properly conducted, the current portfolio of predictive skin sensitization methods, and notably the well-validated LLNA, provides a reliable screen for contact allergens. The LLNA also provides valuable information on potency, so that risk assessment and risk management can be properly implemented on a sound evidential basis (Basketter et al., 2000, 2005). Obtaining equivalent information using a combination of in silico and/or in vitro procedures presents substantial challenges (Jowsey et al., 2006).

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Skin sensitization—A regulatory overview

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1. Introduction

A steadily increasing number of substances are predicted to have the potential to cause allergic contact dermatitis in humans, based on their proving positive in skin sensitization tests conducted in animals for regulatory purposes. In some testing regimes, for example, European Union (EU) new substances legislation, the proportion of positive substances is as high as 30%. Such substances have to be classified, risk assessed, and risk managed as skin sensitizers without much scope for distinction between those that are highly potent and those substantially less so. Recently, however, the local lymph node assay (LLNA) has been accepted internationally as an alternative to the guinea pig methods and, among other significant advantages, offers the opportunity to rate substances according to the potency of any sensitizing effect. This potency information would allow the refinement of risk assessments and so enable the most appropriate level of regulatory control to be assigned.

2. Main points

Currently, in the EU, a technical guidance document (TGD) on risk assessment (EC, 2003) is widely used in support of the regulations on new chemicals, existing chemicals, and biocides. This document describes the different sorts of information that may be used to assess the potential of a substance to cause skin sensitization, points to take into account when evaluating these various data, which types of studies provide dose–response data and potency information, and the degree of uncertainty in studies of sensitization.

The definition of a skin sensitizer given in the TGD is "an agent that is able to cause an allergic response in susceptible individuals. The consequence of this is that following subsequent exposure via the skin the characteristic adverse health effects of allergic contact dermatitis may be provoked."

2.1. Hazard assessment

The general objectives are to find out whether there are indications from human experience of skin allergy following exposure to the agent and/or whether the agent has skin sensitization potential based on tests in animals (EC, 2003).

2.1.1. Human data

Case studies or epidemiological data may be available from human exposure, particularly in the case of existing substances and biocidal products. Data from diagnostic clinical studies (e.g., patch tests) are also sometimes available.

2.1.2. Animal studies

There are three predictive test methods currently described in EU Annex V (EC, 1984) and Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 1992, 2002) for skin sensitization in animals:

1. the guinea pig maximization test (GPMT)—an adjuvant method;
2. the Buehler test—a non-adjuvant method in guinea pigs; and
3. the murine local lymph node assay (LLNA).

Other studies performed in animals may be available and give some information on the sensitization potential of a substance. Some information can be obtained from consideration of structure–activity relationships and comparison with structures of known sensitizers. Validated in vitro methods for sensitization testing are not yet available for regulatory purposes (EC, 2003).

For many substances assessed under the regulatory schemes, human data are generally absent, sparse, or very difficult to interpret. The EU TGD (EC, 2003) does give details on the points to which attention should be paid when evaluating human data.

Historically, for regulators in the EU, the preference has been for the GPMT. However, experience in the United Kingdom under the Notification of New Substances Regulations (NONS) has been that this test is often unsatisfactory for assessing new chemicals. The LLNA has been shown to have clear animal welfare and scientific advantages compared with guinea pig tests, and the LLNA has consequently been adopted within the EU as the method of choice. Experience in the United Kingdom of assessing test reports for submissions under NONS has

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shown the LLNA to be a successful replacement for the guinea pig tests (Cockshott et al., 2006).

In 2007, new EU legislation, REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), is expected to come into force, replacing the new (NONS) and existing chemicals regulations. As stated in the draft regulations (Council of the European Union, 2006), where an assessment of skin sensitization is required, that assessment shall comprise two consecutive steps:

1. an assessment of the available human, animal, and alternative data; and
2. in vivo testing.

However, step 2 does not need to be conducted if the available information indicates that the substance should be classified for skin sensitization or corrosivity; or the substance is a strong acid (pH <2) or base (pH >11.5); or the substance is flammable at room temperature. If in vivo testing is necessary, then "the murine LLNA is the first-choice method for in vivo testing. Only in exceptional circumstances should another test be used. Justification for the use of another test shall be provided."

2.2. Classification and labelling criteria

According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nations, 2005), substances should be classified as contact sensitizers (category 1) in accordance with the criteria below:

- if there is evidence in humans that the substance can induce sensitization by skin contact in a substantial number of persons; or
- if there are positive results from an appropriate animal test.

Specific considerations to be taken into account when assessing the data are given.

Under both the current EU and the GHS classification schemes, a substance is either classified as a skin sensitizer or not classified; there is no differentiation as to strength of sensitization effect.

Many chemicals are supplied on the market as preparations or mixtures, and often there are no data on the mixture as a whole but only on one or more of the components. In this case, unless there are specific concentration limits for a particular chemical, a mixture containing a known skin sensitizer as an ingredient at a concentration greater than or equal to 1% requires classification and labelling as a potential skin sensitizer. As people who are already sensitized to a substance may be affected by very small amounts of it, in the EU, the label on the packaging of preparations containing at least one substance classified as sensitizing and being present in a concentration equal to or greater than 0.1% or in a concentration equal to or greater than that specified under a specific note for the substance in Annex I to Directive 67/548/EEC (EC, 1984) must bear the inscription: "Contains (name of sensitizing substance). May produce an allergic reaction" (EC, 1999).

Currently, this default position may be inadequate for strong sensitizers where evidence for high potency would indicate the need for particularly stringent control or even prohibition in certain circumstances, such as use by consumers. Conversely, this default is likely to be unnecessarily conservative for less potent sensitizers.

2.3. Measurement of dose–response and potency

It is frequently difficult to obtain dose–response information from either existing human or guinea pig data where only a single concentration of the test material has been examined (EC, 2003). With the dose–response data being generated by the LLNA, there is the possibility of using the EC3 value as a measure of relative potency (ECETOC, 2000), and the potential to classify skin sensitizers according to potency has also been evaluated (ECETOC, 2003; Basketter et al., 2005).

An internationally accepted set of principles could allow more refined judgements to be made, incorporating distinctions based on potency. General agreement on such approaches could lead to a significantly better control situation in which risk and risk management measures are better tailored to the degree of threat.

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Quantification, potency, and risk assessment: Induction versus elicitation

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1. Background

Allergic contact dermatitis (ACD) develops in two distinct phases. In the induction phase, a naive individual is exposed to an amount of chemical allergen over a defined surface area of skin (dose per unit area) that is sufficient to provoke a cutaneous immune response of the magnitude necessary for a degree of immunological priming (systemic sensitization). The elicitation phase occurs upon a subsequent exposure to the same chemical in an amount sufficient to provoke a dermal inflammatory reaction—the clinical symptoms that are recognized as ACD (reviewed in Basketter et al., 1999).

Dose–response relationships can be observed for both the induction and elicitation phases of skin sensitization (Kimber et al., 1999). They are considered to be threshold phenomena; as such, a level of chemical exposure can be determined below which sensitization will not be induced or below which an allergic response will not be elicited in a sensitized individual. However, for any given allergen, those thresholds are not absolute values and may not be applicable to a population. Therefore, it is important to have an appreciation of the differences between induction and elicitation thresholds among individuals and the factors affecting them.

2. Main points

2.1. Factors that influence thresholds (both induction and elicitation)

A number of factors can impact the threshold for the induction of sensitization (Basketter et al., 2002). One such factor is the amount of allergen that is delivered to the skin. Usually in skin sensitization studies, the treatment dose or topical exposure is recorded as the concentration of chemical expressed as a percentage, often weight per volume or volume per volume. This would lead one to believe that the same amount of chemical allergen (i.e., concentration) would induce a similar level or frequency of sensitization, regardless of exposure conditions, including the area of skin exposed. However, there is overwhelming evidence derived from both human and experimental animal data that, under most normal conditions of exposure, it is the dose of chemical per unit area of skin that is the key metric in terms of the acquisition of skin sensitization. The importance of dose per unit area, usually reported as micrograms of chemical per square centimetre of exposed skin, is perhaps best illustrated in the work conducted in human subjects by Peter Friedmann and his colleagues in the 1990s (Friedmann, 1990, 1996) and Albert Kligman in the 1960s (Kligman, 1966a).

Another key influence on induction thresholds is the inherent potency of the allergen (Kimber et al., 2003). In general, the more potent the allergen, the lower the dose per unit area required to induce sensitization. This point can be easily demonstrated by using local lymph node assay (LLNA) EC3 values (effective concentrations inducing a stimulation index of 3) to compare the relative potencies of contact allergens. While reported as a percent concentration, EC3 values can be converted to the relevant dose metric using the fact that the application of 25 μl of a 1% solution results in a dose per unit area of 250 $\mu g/cm^2$. For example, the EC3 value for isoeugenol, a sensitizer of moderate potency, is 1.3% (325 $\mu g/cm^2$), whereas the EC3 value for hydroxycitronellal, a weak allergen, is 20% (5000 $\mu g/cm^2$) (Kimber et al., 2003). Thus, a greater amount of allergen per unit area of skin is required to induce a threshold response.

The vehicle matrix in which the skin encounters the chemical allergen can also affect the sensitization threshold (Kimber et al., 1999). Exposure conditions such as the duration and frequency of contact and occlusion are also influencing factors. The presence of inflammation can also impact sensitization thresholds (McFadden and Basketter, 2000).

It is reasonable to assume that all of the above-mentioned factors that can influence induction thresholds can also affect elicitation thresholds.

2.2. Variation of thresholds for induction and elicitation between individuals

At the induction stage, the variability in thresholds among subjects to any given allergen may be due to exposure conditions such as those indicated above or to interindividual differences in inherent or acquired susceptibility to

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sensitization. There is evidence to support the hypothesis that some individuals may be more susceptible to the development of ACD. Brasch et al. (2006) noted that patch test patients who responded strongly to nickel or fragrance mix were more likely to have positive reactions to unrelated contact allergens, suggesting that there is a population of individuals who, owing to some yet-to-be-identified factor(s), are predisposed to the acquisition of skin allergies. In addition, genetic differences among individuals, such as polymorphisms in genes for skin metabolizing enzymes (Kawakubo et al., 2000; Nacak et al., 2006) and cytokines (Dai et al., 2004), have been linked to increased susceptibility to the development of ACD in response to specific chemicals.

At the elicitation stage of contact allergy, one important factor in threshold variation between individuals is the extent to which sensitization was acquired previously. Generally, it is recognized that the lower the dose of chemical used for induction, the higher the dose of the same chemical that will be required to elicit a contact allergic reaction in the sensitized subject (Friedmann et al., 1983; Friedmann, 1990; Scott et al., 2002; Hostynek and Maibach, 2004).

2.3. Relative ease of identification of thresholds for induction and for elicitation

The LLNA directly examines events that are associated with the induction phase of the allergic response; there is no elicitation or challenge phase in the assay, as is the case for the standard and accepted guinea pig methods (e.g., the guinea pig maximization test or the Buehler test). Some attempts have been made to redesign guinea pig tests for the purpose of deriving induction dose–response information (Andersen et al., 1995). However, among the various animal tests available, the LLNA, by derivation of EC3 values, is the method of choice for identifying thresholds for induction (Kimber et al., 2003).

For ethical reasons, test procedures should not be conducted in humans for the sole purpose of identifying induction thresholds. However, the published literature contains valuable reports of dose–response induction studies that have been conducted in humans via non-diagnostic human repeat patch testing, including both the human maximization test (Kligman, 1966b) and the human repeat insult patch test (Marzulli and Maibach, 1974).

Using a population of sensitized individuals, thresholds for elicitation can be defined under 48-h occluded diagnostic-type patch tests with serial dilutions of the allergen (e.g., Johansen et al., 2003). Repeat open application tests (Hannuksela and Salo, 1986) provide somewhat more realistic exposure conditions relative to consumer product exposures and have also been used to define elicitation thresholds for various contact allergens, such as formaldehyde (Flyvholm et al., 1997), isoeugenol (Johansen et al., 1996a), and cinnamic aldehyde (Johansen et al., 1996b). Regardless of the methodology used, as mentioned previously, the elicitation threshold as defined in any individual will depend on the exposure conditions under which sensitization was acquired.

2.4. Relationship between induction thresholds and elicitation thresholds

Attempts have been made to describe a quantitative relationship between induction and elicitation thresholds (Nakamura et al., 1999; Scott et al., 2002). A key observation, based on a number of studies conducted across species, is that the threshold for elicitation is not an inherent property of an allergen, but is a consequence of the severity of the induction regime (Hostynek and Maibach, 2004). In general, the amount of chemical required to induce sensitization is usually greater than the amount of the same chemical needed to elicit a response in a sensitized subject. As well, as indicated previously, the amount of chemical required to elicit a response decreases as the induction dose increases.

3. Conclusions and future directions

In summary:

- The relevant dose metric for describing the amount of allergen that is delivered to the skin is the dose of chemical per unit area of skin (i.e., $\mu g/cm^2$).
- Thresholds for both the induction of skin sensitization and the elicitation of ACD can be identified.
- A number of factors can affect these threshold values.
- Threshold values for induction and elicitation can vary between individuals.
- Thresholds for elicitation are dependent, to some extent, upon the exposure conditions during the acquisition of sensitization.

Clearly, the future direction will be to apply our knowledge of the mechanisms associated with skin sensitization to reduce the occurrence of ACD. The most effective strategy to control the elicitation of ACD is to prevent the induction of skin sensitization in the first place. This is the aim of the exposure-based quantitative risk assessment approach, which relies on the identification of a weight-of-evidence no-expected-sensitization induction level (i.e., the induction threshold) for the chemical in question and an accurate determination of the anticipated consumer exposure to it.

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Uncertainty factors and risk assessment for skin sensitizers

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1. Introduction

A few decades ago, allergic reactions to chemicals were often regarded as inaccessible for quantitative risk assessment (QRA) and were seen as all-or-none responses lacking dose–response relationships and thresholds. This was probably a result of how the immune system works: its response is characterized by a "learning phase" without symptoms (termed primary immune response or sensitization phase or induction), followed by the immune response effector phase (termed secondary immune response or elicitation phase or challenge reaction). Consequently, the first contact (and often repeated contacts), even with relatively high concentrations of a sensitizer, can go unnoticed, because no signs or symptoms of allergy occur. Nevertheless, this contact may induce sensitization, that is, cause the immune system to prepare for a reaction at the next contact. Once sensitization is established, every contact with the same sensitizer—sometimes even at concentrations several orders of magnitude lower—will lead to symptoms of allergic contact dermatitis (for further reading, see references cited in the contribution by G.F. Gerberick).

In the realm of chemical regulation, current risk management measures (e.g., classification and labelling and requirement for personal protection measures) are mostly based on the classification of chemicals and mixtures/formulations into either sensitizers or non-sensitizers. Recently, suggestions for classification systems using sensitization potency categories have been put forward (see, for example, EC, 2003; ECETOC, 2003; Akkan et al., 2004; Schneider and Akkan, 2004; Basketter et al., 2005a).

From basic immunological research and experimental studies in animals and humans, we know today that skin sensitization as well as allergy elicitation occur only above threshold doses and follow predictable dose–response relationships (see, for example, Kimber et al., 1999; Boukhman and Maibach, 2001; and references cited in the contribution by G.F. Gerberick). It has been shown that skin sensitization thresholds for different chemicals are spread over at least 5 orders of magnitude. This wide range of sensitizing potency suggests that solely hazard-based risk management may not be the most adequate form of addressing skin sensitization risks, especially because skin contact with potential sensitizers, for example, from consumer products and at the workplace, cannot be avoided completely. An exposure-based QRA to determine safe exposure levels of skin sensitizing chemicals may be better suited for setting exposure levels with negligible risk, for identifying safer alternative substances, and for protecting the health of workers and consumers.

2. Main points

In principle, the skin sensitization QRA approach follows the same four fundamental steps as identified for general toxicology risk assessment: hazard identification, dose–response assessment, exposure assessment, and risk characterization.

2.1. Hazard identification

Hazard identification either is based on human experience or involves the use of experimental data to determine the skin sensitization potential of a substance. Typically, the murine local lymph node assay (LLNA) or the guinea pig maximization test (GPMT) is used. The contribution by G. Patlewicz explores the possibilities of using structure–activity relationships. Criteria used to classify a substance as skin sensitizing have been published, for example, in the European Dangerous Substances Directive 67/548/EEC (EC, 1984), in the Globally Harmonized System of Classification and Labelling of Chemicals (United Nations, 2005), and by ECETOC (2003).

2.2. Dose–response assessment or hazard quantification

2.2.1. Dose metric for induction and elicitation of skin allergy

Convincing evidence (reviewed, for example, in QRA Expert Group, 2006) suggests that the adequate dose metric for skin sensitization is the skin area dose—that is, the amount of chemical (remaining on the skin, for example, after rinse-off) per unit area of skin, expressed as nanomoles or micrograms per square centimetre. Multiple exposures onto the same skin area can be taken into account by using the cumulative area dose per day ($\mu g/cm^2$ per day).

The effectiveness with which a chemical can cause skin sensitization depends on a number of factors. Of prime importance is the skin penetration of the substance, that is, the topical dose versus the dose delivered to the first

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layers of living cells in the skin. Besides skin penetration, other factors, such as evaporation, metabolism on/in the skin (either inactivation or activation), sequestration in the stratum corneum, binding to protein or cells in the epidermis, as well as uptake and presentation by antigen-presenting cells and recognition by T-lymphocytes, determine if and how strong an immune response is triggered (reviewed, for example, in Kimber et al., 1999; Boukhman and Maibach, 2001; Gerberick et al., 2001a; Griem et al., 2003; QRA Expert Group, 2006). Typically, there is very little information available about the bioavailability (here, the availability to cells of the immune system) of sensitizing chemicals in either the experimental situation or real-life exposure scenario. Therefore, it is suggested (QRA Expert Group, 2006; see also contribution of G.F. Gerberick) that the applied area dose be used as a dose metric and that the uncertainty in this area be accounted for by the use of uncertainty factors (more precisely, all the parameters mentioned above are implicitly covered as part of uncertainty factors for differences between species, individuals, chemical matrices in which sensitizers occur, and use regimes).

2.2.2. Induction

Typically, the dose–response for induction of skin sensitization is determined in the first instance using animal assays such as the LLNA. Confirmatory human assays, such as the human repeat insult patch test (HRIPT), may be subsequently conducted for substances intended for skin contact to provide substantiation of the LLNA data (see contribution by A.M. Api). The aim is to define a point of departure for risk assessment. For ethical reasons, no-observed-effect levels (NOELs) or benchmark doses (BMDs) from studies in humans are normally not available. Therefore, in a number of studies, human NOELs and BMDs were compared with LLNA thresholds (EC3 values, or the effective concentrations inducing a stimulation index of 3), and it was found that the average ratio of both values is close to 1, indicating that area doses are directly comparable between mice and humans—that is, a sensitization threshold of 10 μmol/cm^2 in mice corresponds to a NOEL or BMD of 10 μmol/cm^2 in humans. Therefore, the LLNA EC3 value has been suggested as a surrogate NOEL in QRA (Basketter et al., 2000, 2005b; Gerberick et al., 2001a,b; Griem et al., 2003; Schneider and Akkan, 2004). For certain substances that are intended to come into contact with the skin of consumers, such as cosmetic ingredients, confirmatory HRIPTs using an area dose not exceeding the area dose equivalent to the LLNA EC3 may be acceptable (Api, 2002; QRA Expert Group, 2006).

Guidelines to apply a weight-of-evidence approach to all available human and animal data in order to derive a point of departure for the QRA have been suggested for fragrance ingredients (QRA Expert Group, 2006). This group suggested naming the point of departure the "no-expected-sensitization induction level" (NESIL).

2.2.3. Elicitation

The dose–response for elicitation of allergic contact dermatitis can be determined in different experimental setups. In clinical patch tests on allergic patients, the concentration of the sensitizer (in a suitable vehicle such as Vaseline) can easily be varied and an elicitation threshold determined. Alternatively, the repeated open application test (ROAT) or a product use test can be employed. The patch test minimum elicitation threshold (MET), for example, as the MET inducing a threshold response in 10% of the subjects tested (MET_{10}), and a NOEL or BMD from a ROAT or use test have been proposed as points of departure for risk assessment (Weaver et al., 1985; Sosted et al., 2006; Zachariae et al., 2006; see also contribution of G.F. Gerberick).

The elicitation thresholds are usually determined in subjects who have had an established allergy for a long period of time. Tests in which elicitation thresholds were obtained using newly sensitized subjects (e.g., in the human maximization test [HMT] and HRIPT) showed that elicitation thresholds in these subjects depend on the sensitization dose used; that is, the higher the sensitization dose, the lower the elicitation threshold (Friedmann et al., 1983). This dependency has also been found in mice (Scott et al., 2002). Thus, it seems that the elicitation threshold decreases with the time of established allergy and with the number of exposures. Although it has not been formally shown that a "minimum threshold" is finally approached, the thresholds determined in well-established allergic individuals seem more reliable than those determined after experimental sensitization.

2.3. Exposure assessment

Exposure to the skin sensitizer is determined using habits and practice data for products containing the substance and may be complemented by experimental measurement of skin exposure. While the importance of the exposure assessment for an adequate risk characterization cannot be overestimated, it is beyond the scope of this contribution to provide an overview of this topic.

2.4. Risk characterization

An extrapolation/uncertainty factor approach can be applied to the selected point of departure in order to derive an acceptable level of exposure to a skin sensitizing substance. It has been proposed to term this factor "sensitization assessment factor" (SAF) (QRA Expert Group, 2006). The acceptability or unacceptability of the real-life exposure situation with respect to sensitization induction or allergy elicitation can then be determined accordingly. To this end, the point of departure for risk assessment (for either induction or elicitation), expressed as area dose, is divided by the SAF to derive an acceptable exposure level. An estimated/determined exposure, expressed as area dose, below this acceptable exposure level is then considered

without appreciable risk of, respectively, sensitization of non-sensitized subjects and elicitation of acute contact dermatitis in already sensitized subjects. The SAF is calculated by multiplication of individual factors that account for interspecies and intraspecies variability as well as for matrix and use.

2.4.1. Interspecies factor

Comparison of human NOELs with LLNA EC3 values suggested that a factor of 3 ($10^{0.5}$) is sufficient to cover the species variability (Griem et al., 2003), especially since vehicle differences in the human and animal exposure are also taken into account in the matrix factor. The interspecies factor can be set to 1 if the point of departure is based on human data. This applies to both induction and elicitation.

2.4.2. Intraspecies factor (interindividual variability)

This factor accounts for possible variations in the sensitivity between individuals due to factors such as genetic effects, higher susceptibility (e.g., individuals with multiple skin allergies or those with damaged skin from pre-existing skin disease), decreased inherent barrier function, age, gender, and ethnicity. These contributing factors have been discussed, for example, by Felter et al. (2002), Griem et al. (2003), and QRA Expert Group (2006). For induction, a factor of 10 has been proposed to adequately cover interindividual variability. With regard to elicitation, there is a considerable variation of the NOEL and MET both between individuals and when the test is repeated in the same individual (Jerschow et al., 2001). While this could be an argument for applying a default factor of 10, it should also be considered that the point of departure used for risk assessment is already based on the lowest MET, that is, the most susceptible individuals.

2.4.3. Matrix factor

The matrix factor has been introduced in the safety evaluation concept for sensitizing fragrance ingredients in cosmetic products (Felter et al., 2002; QRA Expert Group, 2006). Consideration of matrix effects encompasses extrapolation from the matrix/vehicle used to determine the EC3/NOEL in the experimental situation to the product formulation containing the fragrance ingredient to which the consumer is exposed in real-life scenarios. The larger the difference between the experimental situation and real-life exposure scenario, the greater the factor will be. The two areas within vehicle/matrix effects that are especially noteworthy are irritants and penetration enhancers. Usually a value of 1, 3, or 10 is chosen for the matrix factor.

2.4.4. Use factor

The QRA Expert Group (2006) considered three key parameters when extrapolating from the controlled experimental situation (either human or animal) to the real-life scenario. These are site of contact, dermal integrity, and occlusion. The larger the difference in skin site location (e.g., compared with the test site, skin may be more easily irritated, highly follicular, or shaved), effect on barrier integrity (e.g., from diaper rash, existing dermatitis, wet work), and occlusion (e.g., from diapers, gloves, or axillary products), the greater the factor. Usually a value of 1, 3, or 10 is chosen for the use factor. As a fallback for situations in which the use scenario is unknown or cannot be accurately described, application of a repeat exposure factor of 10, instead of the use factor, has been suggested (Griem et al., 2003).

2.5. Examples of risk assessments

- Cosmetic ingredients (e.g., fragrance ingredients and preservatives) (Gerberick et al., 2001a; Felter et al., 2002; QRA Expert Group, 2006), as well as hand wash detergents and fabric softeners (Schütte and Kern, 2005)
  Endpoint: induction
  Point of departure: confirmatory HRIPT NOEL based on LLNA EC3
  SAF: interindividual factor (10) × matrix factor (1–10) × use factor (1–10)
- Sensitizing chemicals in general (Griem et al., 2003)
  Endpoint: induction
  Point of departure: HRIPT NOEL, HMT NOEL, or LLNA EC3
  SAF: interspecies factor (3) × interindividual factor (10) × repeated exposure factor (10)
  Endpoint: elicitation
  Point of departure: patch test NOEL
  SAF: interindividual factor (10) × repeated exposure factor (10)
- Metals in household consumer products (Basketter et al., 2003)
  Endpoint: induction
  Point of departure: LLNA EC3
  SAF: interspecies factor (1) × interindividual factor (10) × matrix factor (1–10) × use factor (1–10)
- Hexavalent chromium (Nethercott et al., 1994)
  Endpoint: elicitation
  Point of departure: patch test 10% MET
  SAF (not explicitly stated, but implicitly used for deriving acceptable Cr(VI) concentration in soil): intraspecies factor (1) × matrix/vehicle factor (1)
- Pesticides (hexavalent chromium) (U.S. EPA FIFRA-SAP, 2004)
  Endpoint: induction
  Point of departure: human NOEL (LLNA EC3 seen as promising)
  SAF: (interspecies factor (1–10) ×) intraspecies factor (1–10) × matrix/vehicle factor (1–10) × repeated exposure factor (1–10)
  Endpoint: elicitation
  Point of departure: patch test 10% MET or ROAT 10% MET (as BMD_{10})
  SAF: intraspecies factor (1–10) × matrix/vehicle factor (1–10) × exposure factor (1–10)

3. Conclusions and future directions

Risk assessment of skin sensitizers is not principally different from that for other toxicological endpoints. Both induction of sensitization and elicitation of allergic responses follow dose–response relationships and show thresholds below which no reactions occur. The main difference between sensitization and systemic toxicity endpoints is that for skin sensitization, the adequate descriptor of exposure is dose per skin area, expressed as nanomoles or micrograms per square centimetre per day. The extrapolation/uncertainty factor approach can be used to derive acceptable non-sensitizing and non-eliciting area doses for induction and elicitation, respectively. However, up to now, this concept has been used in isolated cases and for limited, well-defined fields of application. The concept might gain and be improved through discussion involving all stakeholders (academia, industry, clinic, authorities) of issues such as points of departure for risk assessment, extrapolation/uncertainty factors, fields of application, and regulatory implications.

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Hazard identification (human data)

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1. Introduction/background

Skin sensitization is a result of a series of immunological events following skin contact with a substance of low molecular weight, for example, in cosmetic products or at work. In this process, the immune system is specifically triggered, and permanent changes occur.

Continued exposure or re-exposure to the substance in question in sufficient concentrations will give rise to disease symptoms: erythema, oedema, scaling, and possibly vesicles, that is, allergic contact dermatitis. Allergic contact dermatitis most frequently affects the hands and face, but might be generalized. It has a propensity to become chronic, and the consequences for the individual may be work incapacity, lifelong treatment to reduce symptoms, and reduced quality of life (Frosch et al., 2006).

Population-based studies show that about 20% of the general population is sensitized to one or more allergens (Nielsen et al., 2002). Skin sensitization is a predominantly environmental disease, and the total number sensitized in a given population reflects the exposure level(s). This means that skin sensitization can be prevented by identifying skin sensitizers and reducing exposures to acceptable levels (Johansen et al., 2006).

Skin sensitization is a specific immunological event, which can be identified by a biological test, the patch test. The patch test is an internationally standardized method that is used worldwide for diagnosing skin sensitization. The outcome of the patch test together with clinical information and exposure analysis can be used to establish a causal link between exposure to a given substance and the effect: skin sensitization.

In hazard identification, a causal link is established between exposure to a given substance and an adverse health effect. It is the basis of risk assessment/risk management; only if a hazard is correctly identified is it possible to perform risk assessment and risk management. Several examples exist where hazard identification using predictive test systems has failed, and the uncontrolled exposure of the population that followed caused epidemics in the workplace or among consumers (Menné and Wahlberg, 2002; Thyssen et al., 2007).

Two kinds of human data exist:

1. experimental induction of skin sensitization in healthy volunteers; and
2. clinical evidence of skin sensitization from patients with allergic contact dermatitis.

1.1. Experimental induction of skin sensitization in healthy volunteers

In experimental studies using humans, induction is performed deliberately by exposing healthy individuals to potential contact allergens under exaggerated circumstances. Variations in these methods exist, but in all cases repeated occluded exposures are used and challenge by patch testing after a latency period is performed. The most known methods are the human maximization test (HMT) and the human repeated insult patch test (HRIPT) (Marzulli and Maibach, 1974; Kligman and Epstein, 1975). These methods have also been used to identify skin sensitizers and rank them into groups of different potency.

Experimental induction of skin sensitization in healthy volunteers has both advantages and disadvantages:

- *Advantages:* Exposures are controlled, and several doses can be tested. No interspecies extrapolation is needed.
- *Disadvantages/problems:* Large cohorts of individuals are needed to give a reliable result. Testing is made in supernormal individuals (a selected group of subjects without pre-existing conditions influencing immune response), who (perhaps) are less sensitive to skin sensitization than a normal (''unselected'') population. Disease is deliberately induced in healthy people. For this reason, the advisory of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers to the European Commission (SCCNFP, 2000) has deemed it unethical to perform these tests, which is also in accordance with the guidance in the European Union's (EU) Dangerous Preparations Directive (EC, 1999).

1.2. Clinical evidence of skin sensitization from patients with allergic contact dermatitis

Clinical data are generated from dermatologists who see patients with dermatitis, suspect that a certain substance has caused skin sensitization, which may partly or fully explain the present skin symptoms, and make diagnostic

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investigations pinpointing the causative substances. These data may be published as case reports, as epidemiological studies in, for example, the workplace, or as studies in consecutively patch tested patients. The patients seen by dermatologists and diagnosed by allergy tests (patch testing) can be regarded on a gross level as incident cases. Even small percentages of allergy among patients seen are estimated to amount to a significant number of new cases per year (Schnuch et al., 2002).

The advantages and disadvantages associated with the use of clinical data are as follows:

- *Advantages:* Data represent the effect of a real exposure. No interspecies extrapolation is necessary. Human data are very scarce (luckily) in almost any other area of toxicology. In skin sensitization, such data exist, which gives an obligation to use such data in the optimal way to promote prevention of any further cases.
- *Disadvantages/problems:* Not all investigations are published. Data are not automatically presented to authorities, and generally accepted requirements and guidelines for interpretation in the context of hazard identification need to be established. Previously strict criteria for identification of allergens have been developed under the Nordic Committee on Building Regulations based on clinical data (NKB, 1994). In the validation that followed, well-known allergens such as formaldehyde turned out not to be allergens of importance using the developed criteria (NKB, 1994). It is necessary to establish criteria for hazard identification that are realistic and take into consideration the clinical setting, where data are derived, and at the same time take the uncertainties that exist in any data collection into consideration.

The following case definition is suggested, modified from the EU Dangerous Preparations Directive (EC, 1999) and based on World Health Organization (WHO) criteria developed by an expert panel in 1996 (WHO, 1996):

- The substance has caused skin sensitization in at least one person, who has a (likely) current exposure and a typical clinical presentation of disease.
- Re-exposure to the substance, in concentrations likely to be non-irritating, produces a clear positive response at patch testing.
- The patch test is carried out according to international guidelines and read accordingly.

In addition, in hazard identification based on clinical data, in order to take into consideration any uncertainties in methods, data from more than one case in more than one independent centre are required.

2. Main points

- Induction studies in healthy volunteers are unethical and should not be performed.
- Clinical data are scarce in toxicology and, when present, should be used with priority.
- A case definition that takes the clinical setting into consideration should be used.
- More than one case is required to take any uncertainties into consideration.
- Several examples exist where hazard identification using predictive animal tests has failed and accumulating clinical evidence has not been considered as a basis for hazard identification, leading to epidemics of contact sensitization.
- Positive clinical data should overrule any negative animal/human assays.

3. Conclusions and future directions

- Clinical data that fulfil current standards of investigation, showing skin sensitization in more than one patient in more than one independent centre, are a sufficient basis for hazard identification of skin sensitizers.
- More emphasis should be on clinical data in the future in order to promote prevention.
- Clinical cases of disease overrule negative evidence from any predictive test (in animals or humans).
- Induction studies in humans should not be performed for ethical reasons.

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Clinical experience informing risk assessment

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1. Introduction

Allergic contact dermatitis is a common disease that impairs quality of life and work ability, causes suffering in workers and consumers (men and women as well as children), and results in high costs to society. Allergic contact dermatitis is caused by occupational and non-occupational exposure to skin sensitizers, many of which are ingredients in products on the market. The hands and face are most frequently affected (Kadyk et al., 2003; Belsito, 2005; Frosch et al., 2006).

More than 3700 chemical substances have been identified as skin sensitizers. Epidemiological studies have shown that approximately 20% of the adult general population is allergic to one or more skin sensitizers (Nielsen et al., 2002a; Frosch et al., 2006).

The substances that most frequently cause contact allergy in dermatitis patients are included in the standard series for patch testing. The European standard series includes approximately 30 test substances, whereas the only test panel (TRUE Test®) approved by the Food and Drug Administration in the USA consists of 24 test substances. Thus, only a minority of known skin sensitizers are used for screening patients with contact dermatitis. Adequate diagnosis of patients with contact dermatitis often requires patch testing with products, ingredients, and special test series in addition to the standard series. In large parts of the world, patch testing is not performed at all, because there are a lack of experienced dermatologists and limited or no access to patch test material (Nielsen et al., 2002b; Belsito, 2004; Frosch et al., 2006).

2. Main points

2.1. Prevention of allergic contact dermatitis by information

Labelling of products plays an important role, however limited, in the prevention of allergic contact dermatitis. The majority of individuals with contact allergy and allergic contact dermatitis have not been patch tested; as a result, they do not know to which substance they are sensitive and may not avoid exposing their skin to that substance (Holness and Nethercott, 1991; Kalimo et al., 1997; Frosch et al., 2006).

Very few chemical products, consumer products, and products intended for professional use have a label with detailed information on ingredients, including skin sensitizers (Lidén, 2001).

Ingredient labelling of cosmetic products, by uniform terminology (International Nomenclature of Cosmetic Ingredients, or INCI) and irrespective of concentration, is required according to the European Union's (EU) Cosmetics Directive (EC, 1976). This is of help to the dermatologist planning patch testing and informing patients on how to minimize allergic contact dermatitis. It is of help to the well-informed dermatitis patient, who knows and understands to which substance he or she is allergic and who wants to avoid skin exposure to the allergen that may cause dermatitis. INCI names on cosmetic products, however, are generally very difficult for the general consumer to read and understand (Elbro, 1996; Agner et al., 1999; Noiesen et al., 2004; Frosch et al., 2006).

Classification of dangerous substances and labelling of preparations involve an evaluation of the hazard in accordance with EU Directives 67/548/EEC (substances) and 1999/45/EC (preparations) (EC, 1984, 1999) and a communication of that hazard via the label. Classification and labelling are useful tools for risk management of chemicals. The classification also has downstream consequences within the EU legislation. Classification and labelling may be much improved, to be more efficient in prevention of sensitization and allergic contact dermatitis (Lidén, 2001).

The current general classification limit (1%) for risk code R43 (skin sensitization) is far too high to prevent sensitization and allergic contact dermatitis from many potent and/or frequent skin sensitizers. Specific classification limits (below 1%) are rare, and no general approach on how to set these limits has yet been adopted. Many important skin sensitizers have not yet been classified with R43 (Lidén, 2001; Basketter et al., 2005).

A proposal concerning more efficient use of animal data on sensitizing potential of chemical substances for classification as skin sensitizers (R43) was presented by the European Commission (EC) Expert Group on Sensitisation. Additional limits for labelling strong and extreme sensitizers and listing the names of classified skin sensitizers on product labels, according to uniform nomenclature, were proposed. Such an approach would contribute significantly to the prevention of allergic contact dermatitis (Basketter et al., 2005).

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2.2. Prevention of allergic contact dermatitis by limitations

Limitation of some important skin sensitizers has been introduced in European legislation for the protection of public health and the health of the individual worker, consumer, and patient by prevention of sensitization and allergic contact dermatitis (Lidén, 2001; Frosch et al., 2006).

The Nickel Directive (EC, 1994) limits nickel in certain items intended for direct and prolonged contact with the skin, similarly to the previous Danish regulation. In Denmark, studies have shown that sensitization to nickel and nickel-related hand eczema have become less frequent as a result of the Danish regulation. Chromium(VI) in cement is limited, following the successful approach in Nordic countries, where contact allergy to chromium(VI) has decreased. When the limitations were introduced, the EC was firm in its position that only limitation should be used, not labelling of nickel in items or chromium in cement (EC, 1994, 2004; Johansen et al., 2000; Nielsen et al., 2002b; Frosch et al., 2006).

The Cosmetics Directive (EC, 1976) lists the preservatives, hair dyes, and fragrance substances that are allowed or are limited or not allowed in cosmetic products owing to the risk of skin sensitization or other health effects. This has been important in the prevention of sensitization to some very potent skin sensitizers (Lidén, 2001; Frosch et al., 2006).

3. Summary

- Contact allergens in preparations/mixtures, chemical products, cosmetic products, and other consumer products have adverse effects on human health by causing allergic contact dermatitis.
- It is generally not possible for the individual worker, consumer, or patient to protect himself/herself from exposure that may cause skin sensitization and allergic contact dermatitis.
- Only a minority of all individuals with contact allergy and allergic contact dermatitis have been adequately diagnosed by patch testing and informed about their condition and preventive measures on how to minimize skin disease.
- For patients diagnosed with contact allergy, the self-management strategy to avoid relapse of allergic contact dermatitis or chronic disease is to avoid further exposure to the substance in question. Ingredient labelling of consumer products may be supportive.
- The self-management strategy requires that patch testing with the substance has been performed, that adequate information has been given, that the patient is motivated and skilled to implement the strategy, including that he or she has the capability of understanding the information on the label, and that products are properly labelled.

4. Conclusions and future directions

- There is a need to support development of health care, so that individuals with contact dermatitis will have access to adequate treatment, including diagnosis by patch testing and information about how to minimize allergic contact dermatitis.
- There is a need to further develop the legal requirements concerning information on skin sensitizers in different product types intended for consumer and professional use.
- Clinically relevant limitations of some skin sensitizers causing significant health problems have been successful for the prevention of contact dermatitis. The approach should be further developed, implemented, and validated.

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Perspective of the U.S. Environmental Protection Agency's Office of Pesticide Programs on assessment of dermal sensitization risk using hexavalent chromium as a case study

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Assessment of dermal sensitization hazard in the U.S. Environmental Protection Agency's (EPA) Office of Pesticide Programs (OPP) is currently based on the results of dermal sensitization testing under Office of Prevention, Pesticides and Toxic Substances (OPPTS) Guideline 870.2600 (U.S. EPA, 2003). As stated in the Code of Federal Regulations (40 CFR 798.4100), "Information derived from tests for skin sensitization serves to identify the possible hazard to a population repeatedly exposed to a test substance." Information from this test is qualitatively assessed, and, if appropriate, precautionary language is included on the pesticide label as well as the Occupational Safety and Health Administration's Material Safety Data Sheet. Occupational dermal exposures to known or suspected dermal sensitizing pesticide chemicals can then be dealt with appropriately, through either engineering controls or use of personal protective equipment. Non-occupational exposures can normally be dealt with through precautionary label statements.

It has become apparent in recent years that a labelling approach may not always be adequate to mitigate potential dermal sensitization hazard for pesticide chemicals. This is particularly apparent in the case of treated articles, in which a registered pesticide is incorporated into the article to protect the integrity of the article or substance itself (such as paint treated with a pesticide to protect the paint coating, or wood products treated to protect wood against fungal or insect decay). Under such circumstances, the general population may unknowingly be exposed to a pesticide chemical residue in the treated article. Treated articles do not bear a pesticide label or other means of communication to inform and protect the consumer against potential hazards, including the potential for dermal sensitization.

The OPP was interested in developing the foundation of a scientifically sound approach to quantify the dermal sensitization hazard and associated risk for pesticide chemicals, including pesticide chemicals that are incorporated into other materials (i.e., treated articles).

A meeting was held between the OPP's Antimicrobials Division and the Federal Insecticides, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel on 4–5 May 2004, for the purpose of discussing proposed methodologies for quantification of dermal sensitization hazard and risk. The OPP was interested in obtaining expert advice from the Panel on the following issues: strengths and weaknesses of available methods for measuring induction and elicitation thresholds for allergic contact dermatitis; sensitivity of children to induction and elicitation of allergic contact dermatitis compared with adults; and derivation of "safe" area doses using available local lymph node assay (LLNA) data and human patch test data for hexavalent chromium from a treated wood case study.

As noted in the Panel's final report (U.S. EPA, 2004), no particular method was endorsed for risk assessment related to the identification of thresholds for induction by dermal sensitizing chemicals, but the Panel noted that all relevant data should be incorporated into the weight of evidence. Although ethical issues may limit future human testing, the Panel felt strongly that when human data are available, they should be given primary consideration. The Panel agreed with the OPP that there was no evidence to suggest significant differences in sensitivity of children compared with adults to development of allergic contact dermatitis.

For chromium(VI) in treated wood, considering all of the data made available, the Scientific Advisory Panel identified the study of Nethercott et al. (1994) as the best available regarding quantification of a level of chromium(VI) causing dermal sensitization using a sensitized human study population. The Panel identified the "critical dose (lowest observed adverse effect level [LOAEL]) from the Nethercott et al. (1994) study as 0.088 $\mu g/cm^2$, which the Panel considered to be a conservative safety level". This represented the 10% minimum elicitation threshold (MET) in that study. Using uncertainty factors to account for the areas of inter- and intraspecies variation, vehicle/matrix effects, and exposure considerations (i.e., the use of closed patch tests in the Nethercott et al. (1994) study), the Scientific Advisory Panel recommended a "sensitization Reference Dose" (s-RfD) range of 0.09–0.3 $\mu g/cm^2$ for hexavalent chromium. The Panel concluded that this is a specific reference dose for chromium used in treated wood, and this estimate of an s-RfD should be protective against elicitation (i.e., reactions in already sensitized persons) and therefore would also be protective against induction (i.e., reaction in non-sensitized persons). The Panel

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suggested that a repeat open application test protocol could be conducted to better represent real-life exposures to treated wood containing hexavalent chromium for refinement of this risk assessment.

Subsequent scientific deliberations within the U.S. EPA through the Steering Committee of the U.S. EPA's Science Policy Council resulted in modification of the s-RfD to a single value of 0.009 μg/cm^2, based on the same LOAEL of 0.088 μg/cm^2 from the Nethercott et al. (1994) study and an uncertainty factor of 10 to account for intraspecies variation. It was also concluded at this time that as more data became available, this value could be reconsidered.

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New approaches for skin sensitization hazard identification: (Q)SARs/expert systems

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1. Introduction/background

Non-testing approaches to hazard identification comprise read-across, (quantitative) structure–activity relationships [(Q)SARs], and expert systems. Under the current European Union (EU) legislation for "new" and "existing" substances, the use of (Q)SARs has been limited, probably due to some disagreement in the scientific and regulatory communities over the applications of (Q)SARs and the extent to which (Q)SAR estimates can be relied upon.

Read-across reflects a situation where information on one chemical is related to another on the basis that both are likely to behave in the same way on account of their similarity to each other (where similarity is likely to be a combination of structural and mechanistic features). SARs are typically characterized by structural alerts, that is, fragments within a chemical that are thought to be indicative of toxicity potential. QSARs as defined here are quantitative statistical correlation models that relate a toxicity endpoint to one or more numerical descriptions (so-called descriptors) of a chemical. Expert systems encompass SARs, QSARs, or both. Typically these are commercial systems and are often known as knowledge-based, statistical, or hybrids. Examples of all three types are, respectively, Derek for Windows (DfW) (LHASA Ltd., Leeds, UK, http://www.lhasalimited.org), Toxicity Prediction Komputer Assisted Technology (TopKat) (Accelrys Inc., San Diego, USA, http://www.accelrys.com/products/topkat/), and TImes MEtabolism Simulator (TIMES) (LMC, Bourgas, Bulgaria, http://omega.btu.bg/).

All these types of non-testing approaches are relevant for skin sensitization, although some are more mature in their development than others.

2. Main points

For a chemical to induce skin sensitization, it must overcome a number of hurdles, including the formation of a stable association with a skin protein. This hurdle is thought to be the rate-determining step of induction. Early work carried out by Landsteiner and Jacobs (1936), followed up by others, for example, Dupuis and Benezra (1982), led to the hypothesis that this stable association was a covalent one whereby the skin protein behaved as a nucleophile and the chemical as an electrophile. Work has thus been predominantly focused on identifying the electrophilic characteristics of a chemical and using these to derive structural alerts or to rationalize read-across evaluations.

Some structural alerts have been derived empirically through analysing sensitization data and looking for trends between the chemicals tested. Others have been identified through an understanding of chemical reactivity and assigning chemicals into mechanistic domains, that is, using common electrophilic–nucleophilic reaction pathways such as S_N2, Michael addition, Schiff base, etc. Examples using this type of mechanistic approach include Payne and Walsh (1994), Gerner et al. (2004), Aptula et al. (2005), Aptula and Roberts (2006), and Roberts et al. (2007a).

Much of the early work in QSARs for skin sensitization has focused on using small data sets for particular chemical classes in order to derive "simple" correlative models (see the review of Smith Pease et al., 2003, for examples). Probably the first such model was that of the relative alkylation index (RAI) developed by Roberts and Williams (1982), which has been used to explore QSARs for many types of chemicals. It continues to form the basis of many of the more recent mechanistic QSAR models being developed. One example is a QSAR developed for Schiff bases (Roberts et al., 2006). Reactivity was encoded using a Taft parameter (Perrin et al., 1981) (measuring the inductive effects of substituents), whereas hydrophobicity was modelled using the octanol/water partition coefficient (log P). While the data set consisted predominantly of aldehydes, the QSAR model itself was able to correctly predict chemicals (such as 1,2-diketones and 1,3-diketones) that were expected to react by the same mechanism. QSARs developed in this fashion are broader in scope and more widely applicable than those focusing on individual chemical classes. These mechanistic QSARs have enabled the prediction of both skin sensitization hazard and relative potency (in cases of local lymph node assay [LLNA] data with defined EC3 values).

There have been and continue to be many efforts to develop QSARs that are statistically driven using large

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data sets of chemicals. Here, the data sets are diverse in chemistry and hence encompass many different mechanisms. They are termed general or global QSAR models. Early work by Cronin and Basketter (1994) found limited success in using such a statistical approach. However, many other workers have tried, by using a host of different descriptors (often starting with 1000 or more descriptors), to identify a handful that correlate well with sensitization using a range of statistical techniques. Examples include Fedorowicz et al. (2004), Miller et al. (2005), and Estrada et al. (2003). In Fedorowicz et al. (2004), the model developed was able to discriminate only between sensitizers and non-sensitizers. In Miller et al. (2005), EC3 values were used to predict potency, although with limited success, and in Estrada et al. (2003), classification bands of potency were used. These approaches have been variable in their successes, and in certain cases the descriptors used have apparently lacked mechanistic insight or physical meaning to help in the interpretation.

Characterization of several of the global statistical models available has been recently carried out (Roberts et al., 2007b) using the Organisation for Economic Co-operation and Development (OECD) validation principles for QSARs (OECD, 2004). These five principles aim to characterize and describe a (Q)SAR in terms of its performance characteristics (predictivity, robustness, and goodness of fit), its transparency in terms of algorithm and underlying data, its mechanistic basis, if available, its applicability domain (scope and limits of a model), and its defined endpoint (to what extent is a (Q)SAR model aiming to predict the outcome from a regulatory assay). In the QSARs investigated in Roberts et al. (2007b), the sensitivities were often found to be quite reasonable, but their specificities tended to be extremely poor.

Expert systems that are available for the prediction of skin sensitization include the three types already described. Perhaps the most commonly used expert system to date is DfW. This is a knowledge base describing the current status of SARs for skin sensitization. Some rules are based on reactive chemistry, whereas others are empirically derived. Other models, such as those in TopKat, are statistically derived from published guinea pig maximization test (GPMT) data predicting classification bands of potency. For each chemical processed, information on whether the prediction is within the scope of the model domain and database is provided. The model also provides a means to search for structurally similar analogues and compare their predicted and experimental data. An example of a hybrid system is TIMES for skin sensitization (TIMES-SS). TIMES-SS encodes structure–toxicity and structure–skin metabolism relationships through a number of transformations, some of which are underpinned by mechanistic three-dimensional QSARs. Thus, TIMES-SS is able to provide an estimate of skin sensitizing potency for both the chemical of interest and any likely metabolites.

3. Conclusions and future directions

The current global statistical models available in the peer-reviewed literature appear to be limited and insufficiently characterized with respect to the OECD validation principles, as evidenced in Roberts et al. (2007b).

The covalent hypothesis has served as and continues to be a most promising way of developing mechanistically based robust QSARs. However, the sensitization data that are available and accessible are limited (in terms of the number of examples that cover the breadth of a given mechanistic domain), suggesting that current efforts to develop new mechanistically based QSARs might soon be exhausted. Focus should be on systematically generating *in chemico* reactivity data to substantiate and confirm mechanistic hypotheses made. This complements efforts in the in vitro field (for examples, see Gerberick et al., 2004; Aptula et al., 2006; Schultz et al., 2006) and should lead to a more integrated means of assessing skin sensitization.

Better descriptors for encoding reactivity are needed, once a chemical reactivity domain has been thoroughly established; approaches to encoding this information in a reusable and automated format that avoids experimental testing need to be considered. Currently, available reactivity descriptors are limited, and this has partly motivated some of the efforts in Schultz et al. (2006).

Metabolism has not been systematically considered. While there have been efforts to compile metabolism data (MDL Metabolite, http://www.mdli.com, and University of Minnesota Biocatalysis/Biodegradation Database, http://umbbd.ahc.umn.edu, are examples of liver metabolism), there has been little focus on addressing skin metabolism. Defining skin metabolic transformations and integrating these with reactive chemistry still need considerable effort.

Defining rules for non-reactive chemicals and chemicals that are unlikely to undergo chemical transformation may also need to be addressed.

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New approaches for hazard identification: The development of in vitro methods for predicting contact sensitization potential

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1. Background

A variety of characteristics determine whether a chemical can function as a contact sensitizer (or allergen), including the ability to penetrate into the skin, react with protein, and be recognized as antigenic by immune cells. The ultimate challenge for developing non-animal test methods for skin sensitization testing will be applying our mechanistic understanding of allergic contact dermatitis (ACD) to the design of predictive in vitro alternative test methods (Ryan et al., 2001). The key determinants for allergenicity—that is, skin penetration, protein/peptide reactivity, and immune recognition—should all be incorporated into an in vitro testing scheme.

Research to develop mechanism-based in vitro methods involves efforts in a number of different areas: (1) modelling of skin penetration of chemicals into viable epidermis/dermis; (2) development of computer-based approaches (e.g., quantitative structure–activity relationships, or QSARs); (3) quantitative measurement of chemical reactivity with peptides/proteins with and without metabolic activation; and (4) development of cell-based in vitro assay(s) to model the ''immune recognition'' of chemical allergens by dendritic cells (DC) and T cells, the key immune cells involved in the induction and elicitation of ACD.

This abstract focuses on two of the above-mentioned areas of effort: (1) the development of methods to examine a chemical's ability to react with a model peptide either directly or after appropriate biotransformation, and (2) the development of cell-based methods to examine a chemical's ability to react with and activate immune cells, specifically DC or cell lines being used as DC surrogates.

2. Main points

2.1. Peptide/protein reactivity

The ability of a chemical or its metabolites to react with protein is one critical event leading to the induction of skin sensitization and the elicitation of ACD. As the correlation between protein reactivity and the ability to induce skin sensitization is well established (Dupuis and Benezra, 1982; Lepoittevin et al., 1998), an in vitro method that assesses the ability of a chemical to bind to a protein or model peptide could be used as a screen for sensitization potential (Divkovic et al., 2005).

In a series of preliminary studies, Gerberick et al. (2004) demonstrated that model peptides containing either cysteine or lysine and glutathione could react with contact allergens and that the degree of reactivity observed correlated with a chemical's sensitization potential. Briefly, in separate reactions, the test chemical is mixed with the two synthetic peptides at 1:10 and 1:50 ratios and with glutathione at a 1:10 ratio. Following a 15-min reaction time for glutathione or a 24-h reaction period for the two synthetic peptides, the samples are analysed by high-performance liquid chromatography and ultraviolet detection to monitor the depletion of glutathione or the peptide following reaction. To date, 82 chemicals (17 strong, 20 moderate, and 15 weak allergens, along with 30 non-allergens) have been evaluated for their ability to react with glutathione or two synthetic peptides containing either cysteine or lysine. The performance of this direct peptide reactivity assay as a method for hazard identification has been evaluated by performing a Cooper statistics analysis. The results of this analysis indicate that the assay has an accuracy of 87.5%, a sensitivity of 90.4%, and a specificity of 83.3%. In addition, good positive and negative predictivity values were achieved, suggesting that the peptide depletion assay performs very well as a screening tool for skin sensitization hazard identification.

Initial interlaboratory studies demonstrated that the assay could be transferred, and a few technical challenges were identified that have subsequently been addressed. A second interlaboratory trial of the direct peptide reactivity assay, which is being coordinated by the European Cosmetic, Toiletry and Perfumery Association (COLIPA), is currently under way.

Some chemicals require metabolic conversion to become protein reactive. Therefore, a second assay system is under development to identify potential haptens that require metabolic activation through oxidation (prohaptens) or are enhanced by an oxidative environment (prehaptens). The peroxidase peptide reactivity assay (PPRA), which uses peroxidase/peroxide as a metabolizing system as a way to

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detect the reactivity of chemical allergens requiring biotransformation, has shown promise.

2.2. Immune cell activation

A number of changes have been reported to occur in Langerhans cells (LC), the principal DC residing in the epidermis, as a result of hapten exposure; cell surface major histocompatibility complex (MHC) class II molecules become internalized (Becker et al., 1992a,b), cell surface marker expression is modulated (Aiba and Katz, 1990; Verrier et al., 1999), and cytokines are produced and released (Enk and Katz, 1992). Given the importance of epidermal LC in the initiation of skin sensitization, it seems appropriate to explore whether there are opportunities to develop alternative approaches to hazard identification based on chemical-induced changes in phenotype or function of these cells. However, since LC constitute only 1–3% of all epidermal cells, human peripheral blood mononuclear cell-derived DC (PBMC-DC) are being used as surrogate LC in the development of in vitro model systems for predictive skin sensitization tests.

To identify novel genes that are regulated following the interaction of haptens with cultured DC, Ryan et al. (2004) examined, at the transcriptional level, the effects of exposure to a contact allergen on DC. Based on the transcript profiling work, quantitative real-time polymerase chain reaction (QRT-PCR) was used to define a list of 29 gene candidates for use in the development of an in vitro assay and analyse them for their predictive potential using a limited chemical training set (Gildea et al., 2006). While many of these genes appear to demonstrate good sensitivity, specificity, dynamic range, and reproducibility, additional testing with an expanded training set is required.

Aeby et al. (2004) reported use of a PBMC-DC test system to assess skin sensitization potential based on the measurement of cell surface CD86 expression by flow cytometry and interleukin (IL)-1β and aquaporin 3 gene expression by QRT-PCR.

A few commercially available human cell lines are being explored as potential surrogates for DC in the development of in vitro methods to identify contact sensitizers. An in vitro method has been described that examines cell surface expression of CD86 and CD54 on THP-1 cells following exposure to contact allergens (Ashikaga et al., 2006). This test, called the human cell line activation test, has been the focus of interlaboratory studies (Sakaguchi et al., 2006) and is currently being evaluated in a ring trial coordinated by COLIPA.

U937 cells are also being considered as DC surrogates. The assay developed by Python et al. (2007) examines cell surface CD86 expression and IL-1β and IL-8 gene expression, whereas the U937 method being assessed in a COLIPA coordinated ring trial looks solely at CD86 expression.

Change in cell surface marker expression on MUTZ-3 cells has been suggested as a model for screening chemicals for sensitization potential (Azam et al., 2006).

3. Conclusions and future directions

Considerable progress has been made on the development of the direct peptide assay, which is suitable for those molecules that either are directly reactive or undergo oxidation or hydrolysis on a time scale that allows interaction with the peptides containing nucleophilic amino acids (e.g., cysteine, lysine). From a hazard identification perspective, this assay demonstrates a high accuracy for distinguishing allergens from non-allergens. To identify potential sensitizers that are prohaptens or prehaptens, a second assay, the PPRA, which uses peroxidase/peroxide as a metabolizing system, is being developed. Initial results are promising, and work to optimize assay conditions is under way.

Much work has been done to exploit DC responses in vitro for the identification of chemical contact allergens. Using various genomics techniques, a panel of genes has been identified that shows potential for use in the development of an in vitro assay for skin sensitization testing. Further evaluation of this panel is needed, and, while it could be accomplished using QRT-PCR, a high throughput screening (HTS) method based on the Luminex® xMAP® technology is currently being explored for this purpose.

Several of the cell lines examined show promise as surrogates for DC in in vitro assays. However, relatively modest changes have been observed in some endpoint measures with potent allergens, so the question of sensitivity of the assays remains. Additional work is needed to support the feasibility of utilizing cell lines as surrogate DC in development of in vitro skin sensitization methods. Another issue that impacts on all in vitro cell assays, including those incorporating DC or DC-like cells, is delivery of the test material. Many of the chemicals to be evaluated are organic in nature and, thus, are insoluble in aqueous culture systems. While aqueous/organic solvent mixtures can be used, this still represents a significant technical challenge.

The ideal in vitro model for a cell-based assay system would be a three-dimensional skin equivalent containing LC or DC. These types of culture systems develop a fully differentiated epidermis with a stratum corneum at the air–liquid interface, which allows for the topical application of test materials and provides a barrier system for skin penetration. These models are currently being developed and represent a significant opportunity for hazard assessment.

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Sensitization potency of chemicals

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1. Introduction

Allergic contact dermatitis caused by chemicals is a serious occupational health hazard. In the past, allergic reactions were regarded as all-or-none responses, implying that the sensitization response is not dependent on exposure dose. However, now we know that the skin sensitization reaction is not different from other toxicological reactions in this respect. This quantitative aspect of the skin sensitization reaction is directly relevant to the sensitization risk assessment in humans. This article comments on the need to predict the sensitization potency of chemicals and to conduct risk assessments based on the sensitization potencies of the chemicals.

2. Prediction of the sensitization potential of chemicals

For a long time, skin sensitization tests using guinea pigs, such as the guinea pig maximization test (Magnusson and Kligman, 1969) and the Buehler occluded patch test (Buehler, 1995), have been used to predict the allergic potential of chemicals. Subsequently, the murine local lymph node assay (LLNA) was developed (Kimber and Weisenberger, 1989), and the test guidelines of this method have been endorsed by several authorities (OECD, 2002; U.S. EPA, 2003). Data obtained from guinea pig predictive tests may be used for prediction of the relative sensitization potency if a suitable experimental design can be employed. However, the guinea pig predictive test methods have limitations in respect of the information that can be obtained with regard to the dose responsiveness and threshold for acquisition of sensitization. Therefore, the LLNA is the recommended method for quantitative assessment of the skin sensitization potential of chemicals, because of the availability of dose-dependent response data and the quantitative parameter, EC3 (the effective concentration inducing a stimulation index of 3).

3. Sensitization potency and sensitization risk in humans

There is no general methodology to determine the absolute sensitization potency of chemicals. Recently, a practical classification of chemicals into five categories according to their sensitization potency was proposed (Table 1; Basketter et al., 2000). This classification, in which 20 chemicals are categorized from Human class 1 (strong human sensitizer) to Human class 5 (non-sensitizer), is based on valuable human data and expert judgment. These are well-known chemicals, and they would be useful as reference allergens to indicate relative sensitization potencies.

On the basis of data from some sensitization predictive tests, the acquisition of skin sensitization is directly associated with the sensitization potency of chemicals; in other words, strong sensitizers can sensitize guinea pigs at very small doses, and weak sensitizers are required in comparably larger amounts to induce sensitization (Table 2 and Fig. 1).

Similarly, comparison of the data from experiments conducted with the same concentrations of chemicals in the non-radioactive LLNA revealed that the extent of the lymphocyte proliferative response was directly associated with the sensitization potency class of the chemicals (Takeyoshi et al., 2005; Fig. 2). Thus, acquisition of sensitization to specific chemicals is closely related to the sensitization potency of the chemicals. In addition to the potency, the level of exposure to the chemicals is also an important factor influencing the acquisition of skin sensitization.

Table 1
Human sensitization potency estimation (Basketter et al., 2000)

Human class	Chemical name	EC3 (%)
1	(Chloro)methylisothiazolinone	0.05
	Dinitrochlorobenzene (DNCB)	0.08
	Diphencyclopropenone	0.05
	p-Phenylenediamine	0.06
2	Cinnamic aldehyde (cinnamal)	2.00
	Glutaraldehyde	0.20
	Isoeugenol	1.30
	Tetramethylthiuram disulfide	6.00
3	Citral	13
	Eugenol	13
	Hexyl cinnamic aldehyde	8
	Hydroxycitronellal	20
4	Ethyleneglycol dimethacrylate	35
	Isopropyl myristate	44
	Propyl paraben	>50
	Propylene glycol	Non-sensitizing
5	Glycerol	Non-sensitizing
	Hexane	Non-sensitizing
	Diethyl phthalate	Non-sensitizing
	Tween 80	Non-sensitizing

>EC3 values obtained from tests conducted in acetone:olive oil (AOO) vehicle.

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Table 2
Typical sensitizers and sensitizing doses in the guinea pig maximization test (GPMT)

Human class	Chemical name	Concentration (%)			Result (GPMT)
		ID	TA	Ch	
1	Dinitrochlorobenzene (DNCB)	0.1	0.5	0.1	+
2	Cinnamic aldehyde (cinnamal)	0.2	2.5	0.75	+
	Isoeugenol	1	25	5	+
3	Eugenol	0.5	100	25	+
	Hexyl cinnamic aldehyde	0.5	50	10	+
4	Isopropyl myristate	100	100	100	+
	Propylene glycol	100	100	100	−
5	Hexane	100	100	100	−

ID, intradermal injection; TA, topical application; Ch, challenge; +/−, positive or negative in the guinea pig maximization test (GPMT).

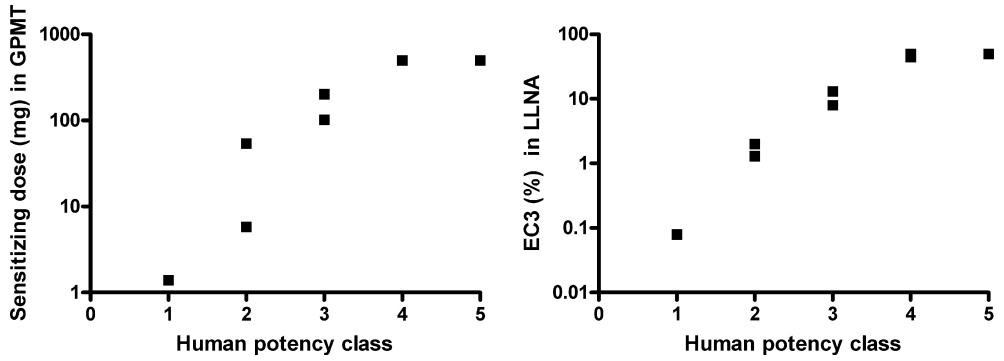

Fig. 1. Relationship between the human sensitization potency class and the sensitizing dose in the guinea pig (left) or EC3 in the murine local lymph node assay (right).

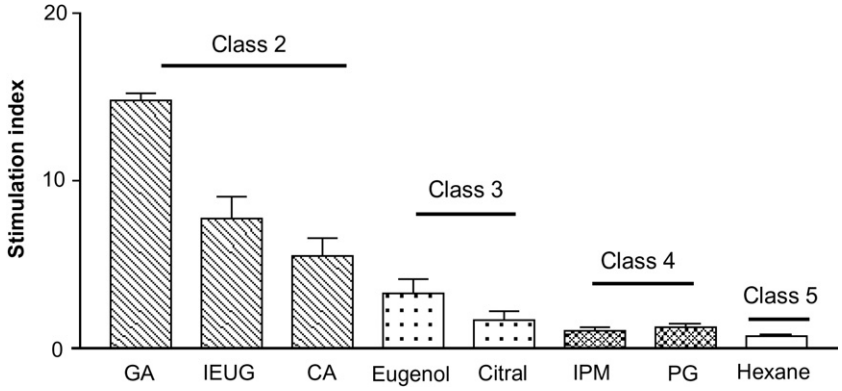

Fig. 2. Lymphocyte proliferative activity of chemicals treated with 10% preparations of chemicals in the non-radioisotopic LLNA. Results are represented as the mean stimulation index and standard error in four animals. *Abbreviations:* GA, glutaraldehyde; IEUG, isoeugenol; CA, cinnamic aldehyde; IPM, isopropyl myristate; PG, propylene glycol.

The sensitization risk can be represented as a function of the sensitization potency and level of exposure to the chemical, as follows:

$$\text{Sensitization risk} = F(\text{Sensitization potency}) * (\text{Exposure level})$$

Accordingly, control of the exposure level is the most effective measure to prevent sensitization to a chemical.

4. Conclusions and future directions

Allergic contact dermatitis caused by chemicals is a serious occupational health hazard. The risk of chemical sensitization in humans depends on the sensitizing potency of and levels of exposure to the chemicals. Thus, the sensitization potency data are absolutely indispensable for estimating the sensitization risk to chemicals, and data on the sensitization potency are expected to contribute greatly to the development of low-risk products in terms of skin sen-

sitization. It is important to estimate the accurate sensitization potency of chemicals and to control the exposure levels in humans. Accordingly, estimation of the risk of sensitization should be conducted based on accurate sensitization potency data and levels of exposure to the chemicals in humans.

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New approaches to hazard identification: Non-radioactive alternatives

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1. Introduction and background

The principle of the local lymph node assay (LLNA) was published in 1989 (Kimber and Weisenberger, 1989), and a first collaborative validation study in 1991 (Basketter et al., 1991). In these initial studies, the stimulation of the lymph nodes, that is, cell proliferation, was measured by [^{3}H]thymidine incorporation. In 1999, the LLNA was accepted by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 1999) as a valid alternative to guinea pig assays, although the need for further modifications was also noted.

The method had already been incorporated into Test Guideline No. 406 (Skin Sensitisation) of the Organisation for Economic Co-operation and Development (OECD) in 1992 and into Test Guideline OPPTS 870.2600 (Skin Sensitization) by the U.S. Environmental Protection Agency (EPA) in 1998. However, according to these guidelines, the LLNA is recommended for the assessment of skin sensitization as an initial screening method; in other words, in case of negative results, additional guinea pig studies have to be conducted. Owing to this limitation, the LLNA was not widely used for skin sensitization studies until 2002, when it was accepted as a stand-alone test by the OECD (Test Guideline No. 429), and a revised EPA guideline was published in 2003 (Test Guideline OPPTS 870.2600, Skin Sensitization).

Some recommendations contained in both guidelines or the ICCVAM report are not always available to everybody or are often debatable. Four points in particular should be mentioned in this connection:

1. The LLNA is the preferred method but should not replace guinea pig methods. Properties of certain test materials may recommend guinea pig assays as the preferred test system.
2. It must be ensured that the test material will not immediately run off. Therefore, wholly aqueous solutions/formulations should be avoided for the LLNA.
3. Although the radioactive method is described as the standard method, other endpoints for assessment of proliferation are acceptable provided there is "justification and appropriate scientific support, including full citations and description of the methodology" (OECD, 2002).
4. There is concern about the influence of irritant properties of the test material, which may cause non-specific cell proliferation in the draining lymph node and thus—in the worst case—lead to false-positive results.

With respect to the first point, there is so much enthusiasm about the advantages of the LLNA compared with guinea pig tests—such as animal welfare refinements, measurement of objective parameters, and short protocol of only a few days—that some of the advantages of guinea pig assays are often ignored. It will take some time for guinea pig assays to be restored to the place they deserve.

Although not the main topic of this contribution, there are also efforts to make aqueous solutions/formulations applicable to the LLNA. Among others, the most prominent modification in this respect to date is the addition of a small amount of detergent to the vehicle, as proposed by Ryan et al. (2002).

This contribution concentrates more on modifications of the radioactive standard protocol directed at incorporation of non-radioactive protocols. However, it also touches on the fourth of the above-mentioned points, that is, the matter of taking account of skin irritation in the evaluation of the data.

2. Main points

From the very beginning, several modifications were published that introduced non-radioactive endpoints to the standard protocol. Some authors simply switched from radioactive labelling of lymph node cells with [^{3}H]thymidine to non-radioactive 5-bromo-2′-deoxyuridine (BrdU) labelling (Suda et al., 2001; Takeyoshi et al., 2001; Piccotti et al., 2006). The overall conclusion by some authors was that this method may not be sensitive enough to pick up all skin sensitizers or that it is at least not as sensitive as the radioactive protocol. Not only for this, but also for several other modifications, the cut-off value as exclusively determined for the radioactive method (EC3, or the effective concentration inducing a stimulation index of 3) was simply applied to the new endpoints (cf. Gerberick et al., 1992; van Och et al., 2000; Piccotti et al., 2006). This overlooks the fact that the EC3 value had been validated and accepted by ICCVAM for the standard protocol used for the LLNA validation, that is, [^{3}H]thymidine incorporation in CBA mice.

However, the cut-off value strongly depends on two parameters: (1) the individual (single animal) variation of

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the endpoint measured and (2) the maximum SI observed for the relevant parameter. Normally, the radioactive method results in relatively high SIs, but also high individual variances if measured on a single animal basis. Based on this, the positive threshold had to be set to an increase in the SI by a factor of 3, that is, EC3. It is obvious that, for each new endpoint, the specific threshold value has to be determined based on the individual variances and the maximum SI that can be expected with the method.

This is very much comparable with the guinea pig situation, where different threshold values have also been defined for different protocols. For example, according to the Buehler protocol, at least 15% of the animals have to respond, whereas the threshold is 30% with the guinea pig maximization test (GPMT). Hence, it is not at all new or unusual to determine specific cut-off values for specific protocols.

Since 1996, alternative protocols have been proposed (Vohr et al., 1994, 2000; Sikorski et al., 1996; Homey et al., 1998) to measure the proliferative activity of draining lymph node cell responses by avoiding radioactivity. Apart from BrdU incorporation, some authors used ex vivo restimulation by T cell mitogen and cytokine expression as endpoints (Suda et al., 2001). In addition, others have sought to characterize draining lymph node cells using flow cytometry (Sikorski et al., 1996; Ulrich et al., 1998; Suda et al., 2001; Gerberick et al., 2002). However, only one of these alternative endpoints has yet been evaluated thoroughly in the context of interlaboratory trials (Ehling et al., 2005a,b). This should therefore be presented in a little more detail. This catch-up validation included a comparison between [^{3}H]thymidine incorporation and simple cell counting to determine SIs after application of internationally accepted standard chemicals. In addition, the acute irritant properties of the standards tested were determined by measuring ear thickness before and ear weight after the animals were sacrificed.

It has been shown by several publications that a modification of the assay by measuring the cell counts instead of radioactive labelling provides comparable sensitivity (Ikarashi et al., 1993; Vohr et al., 1994; Ulrich et al., 1998; Ehling et al., 2005a,b; A.O. Gamer, personal communication). However, this modification has not been formally validated by an international trial. Besides avoiding radioactivity, this modification also has the advantage that the cell suspension can be further analysed by different methods (flow cytometry, chemiluminescence responses, immunofluorescence) to gain an insight into mechanistic events (Vohr et al., 1992, 1994; Ikarashi et al., 1993; Gerberick et al., 1996; Ulrich et al., 2001b). The results confirmed similar sensitivity between both methods, good correlation between participating laboratories, and identical classification of the compounds compared with other methods or human experiences (Schlede et al., 2003).

By comparing the specific immune reaction induced by the test item in the draining lymph nodes (cell counts/lymph node weights) with the immediate nonspecific acute skin reaction (ear swelling/ear weight), it is possible to differentiate the irritant potential from the sensitizing potential of the compound tested. This second modification (measurement of ear swelling/ear weight) after treatment was shown to provide very useful additional information, not only preventing "false positives" from being missed, but also preventing compounds from being wrongly categorized with respect to their skin sensitizing potency (Homey et al., 1998; Blotz et al., 2000; Ulrich et al., 2001a; Ehling et al., 2005a,b). Such modifications are also authorized in the Note for Guidance SWP/2145/00 of the Committee for Proprietary Medicinal Products (CPMP, 2001) and OECD Test Guideline No. 429 (OECD, 2002).

3. Conclusions and future directions

Several modifications of the radioactive protocol of the LLNA have been published so far, but only one of these has been validated by an international trial. From all the data published, it must be concluded that using SI based on cell counts is at least as sensitive as those protocols based on radioactive labelling. Reasonable modifications of the standard protocol of the LLNA will be accepted in the future, as was the case for guinea pig assays previously, especially if such modifications represent real improvements and open the door to obtaining reasonable and valuable additional information from in vivo experiments.

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