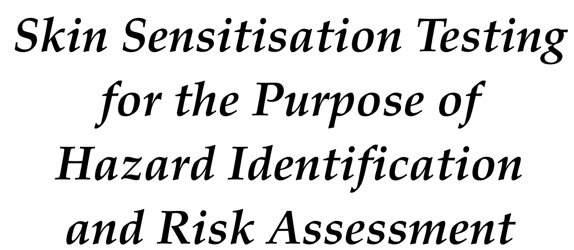

Skin Sensitisation Testing for the Purpose of Hazard Identification and Risk Assessment

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Skin Sensitisation Testing
for the Purpose of Hazard Identification and Risk Assessment

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SUMMARY

In this Monograph various methodological aspects of skin sensitisation testing have been considered and, in addition, the application of certain test methods for the purposes of potency and risk assessment has been explored. The first remit was:

To review relevant skin sensitisation test methods and in the context of animal welfare considerations, make recommendations for the conduct of current and proposed OECD skin sensitisation test methods with respect to (a) appropriate test configuration (protocol) for the purposes of hazard identification and labelling, and (b) the requirement for positive controls.

Specifically, the following aspects of guinea pig sensitisation test methods have been assessed: (1) the numbers of test and control animals required, (2) the option of using joint positive controls between independent laboratories, (3) the choice of positive control chemicals, (4) the optimal conduct and interpretation of rechallenge and (5) the requirement for pretreatment with sodium lauryl sulphate. In addition the use of the local lymph node assay (LLNA) has been considered.

A number of conclusions has been drawn and the following recommendations made:

- In many instances, particularly with the conduct of the guinea pig maximisation test, it is possible to halve the number of test and control animals used and this option should be available to investigators.
- An optional scheme for the conduct of joint positive control studies within a coordinated group of laboratories should be introduced.
- Only one positive control chemical (hexyl cinnamic aldehyde) should be used for the routine assessment of assay sensitivity.
- The proper conduct and interpretation of rechallenge can provide valuable information and confirmation of results in guinea pig sensitisation tests.
- Sodium lauryl sulphate should no longer be used as a pretreatment for the guinea pig maximisation test.
- The LLNA is a viable and complete alternative to traditional guinea pig test methods for the purposes of skin sensitisation hazard identification.

Collectively these recommendations provide opportunities for both animal welfare benefits and improved hazard identification.

The second remit addressed was to :

Make recommendations for the use of relevant skin sensitisation test methods for the purposes of (a) determination of relative potency and the threshold dose necessary for the induction of skin sensitisation, and (b) risk assessment.

In addressing the first part of this remit, the utility of three guinea pig tests (the guinea pig maximisation test, the occluded patch test and the open epicutaneous test), of the LLNA and of human volunteer testing for the assessment of relative potency and identification of thresholds for sensitisation were considered. The following conclusions were drawn:

- Although attempts have been made to reconfigure the guinea pig maximisation test for the purposes of deriving dose-response relationships, this method is usually unsuitable for determination of relative sensitising potency.
- Guinea pig methods that do not require the use of adjuvant and which employ a relevant route of exposure (the occluded patch test and the open epicutaneous test) are more appropriate for the assessment of relative skin sensitising potency.
- The LLNA is suitable for the determination of relative skin sensitising potency and the adaptation of this method for derivation of comparative criteria such as EC3 values provides an effective and quantitative basis for such measurements.
- For all the methods identified above, potency is measured relative to other chemical allergens of known skin sensitising potential. The estimation of likely threshold concentrations is dependent upon the availability of suitable benchmark chemicals of known potency for human sensitisation.
- Human testing (and specifically the Human Repeat Insult Patch Test) can provide information of value in confirming the absence of skin sensitising activity of formulations and products under specific conditions of use and exposure.

Based on the above the following recommendations are made:

- If results are already available from suitable guinea pig tests then judicious interpretation of the data may provide information of value in assessing relative skin sensitising potency. This option should be explored before other analyses are conducted.
- The LLNA is the recommended method for new assessments of relative potency, and/or for the investigation of the influence of vehicle or formulation on skin sensitising potency.
- Whenever available, human skin sensitisation data should be incorporated into an assessment of relative potency.

With respect to the second part of the remit, the conclusion drawn is that all available data on skin sensitising activity in animals and man should be integrated into the risk assessment process. Appropriate interpretation of existing data from suitable guinea pig studies can provide valuable information in consideration of potency as the first step in the development of a risk assessment. However, for *de novo* investigations the LLNA is the method favoured for providing quantitative estimations of skin sensitising potency that are best suited to the risk assessment process. Finally, human testing is of value in the risk assessment process, but is performed only for the purposes of confirming product safety.

GENERAL INTRODUCTION

Skin sensitisation resulting in allergic contact dermatitis is a common health problem. There is a need for improved hazard identification and characterisation of skin sensitising chemicals in order that accurate risk assessments can be derived and appropriate risk management measures implemented. There is available a variety of methods for the prospective identification of skin sensitising chemicals. Historically the species of choice for the toxicological assessment of skin sensitising activity has been the guinea pig. The two methods that have been most thoroughly characterised and most widely applied are the guinea pig maximisation test (Magnusson and Kligman, 1970) and the occluded patch test (Buehler, 1965). Although these methods have served toxicologists well, an increased understanding of the cellular and molecular mechanisms of skin sensitisation, and a willingness to consider species other than the guinea pig, have provided opportunities to consider alternative approaches. In this context two newer methods have been developed using mice. One of these, the mouse ear swelling test (MEST), in common with guinea pig assays, identifies contact allergens as a function of challenge-induced hypersensitivity reactions elicited in previously sensitised animals (Gad *et al*, 1986). The other, the murine local lymph node assay (LLNA), employs a different approach in which skin sensitising chemicals are identified on the basis of their ability to stimulate lymphocyte proliferative responses during the induction phase of contact sensitisation (Kimber and Basketter, 1992; Kimber *et al*, 1994).

Skin sensitisation testing was considered by ECETOC in 1990 (Monograph No 14). Since then, a revised OECD guideline (406) for skin sensitisation has been published (OECD, 1992) and it is timely now to review the most appropriate methods for hazard identification. It is relevant also to ask whether and in what ways the methods available can be employed for evaluating relative skin sensitising potency and in the assessment of risk to humans. Here we have considered those test methods which are recommended in the current OECD guideline as stand-alone assays for skin sensitisation testing (the guinea pig maximisation test and the occluded patch test). Additionally, we have considered one method that is recognised currently in the OECD guideline as a screening test, and for which a draft guideline for its use as a stand-alone method is being prepared (the local lymph node assay) (OECD, 1992). The current validation status of the MEST does not warrant further consideration of this assay in this context.

The Terms of Reference for the Task Force charged with considering these issues were as follows:

Review relevant skin sensitisation test methods and

1. *In the context of animal welfare considerations, make recommendations for the conduct of current and proposed OECD skin sensitisation test methods with respect to (a) appropriate test configuration (protocol) for the purposes of hazard identification and labelling and (b) the requirement for positive controls.*
2. *Make recommendations for the use of relevant skin sensitisation test methods for the purposes of (a) determination of relative potency and the threshold dose necessary for the induction of skin sensitisation and (b) risk assessment.*

A Technical Report (ECETOC Technical Report No. 78, Skin Sensitisation Testing: Methodological Considerations) has already been published which focused exclusively on the first of these remits. The deliberations and recommendations of the Task Force contained within that Technical Report also form Part One of this Monograph. Part Two of the Monograph addresses the second remit.

PART ONE. SKIN SENSITISATION TESTING: METHODOLOGICAL CONSIDERATIONS

Introduction

Test methods for the identification of skin sensitisation hazard have been considered in the context of defining their most appropriate application and the need for the incorporation of positive controls. The methods considered were the guinea pig maximisation test (Magnusson and Kligman, 1970), the occluded patch test (Buehler, 1965) and the murine local lymph node assay (Kimber and Basketter, 1992; Kimber *et al*, 1994). The remit addressed by the Task Force in this first part of the Monograph was:

Review relevant skin sensitisation test methods and

- *In the context of animal welfare considerations, make recommendations for the conduct of current and proposed OECD skin sensitisation test methods with respect to (a) appropriate test configuration (protocol) for the purposes of hazard identification and labelling and (b) the requirement for positive controls.*

1. GUINEA PIG TESTS

1.1 Animal Numbers

1.1.1 Background

Although the OECD guideline recommends the use of 20 test animals and 10 controls, there is a growing consensus that in many instances the use of 10 and 5 guinea pigs, respectively, is sufficient to provide an assessment of skin sensitisation hazard.

1.1.2 Recommendation

It is recommended that in many instances, particularly with conduct of the guinea pig maximisation test, it is possible to halve the number of test and control animals used. This option should be available to investigators.

1.1.3 Rationale

In many circumstances it is appropriate to halve the numbers of test and control guinea pigs, particularly in the guinea pig maximisation test. As such the proposal focuses on the use of 10 test animals and 5 controls. The reason for the selection of these numbers specifically is based upon two considerations. First, that the OECD guideline permits the use of these numbers for identification of a hazard (but not, as is recommended here, for the verification of negatives). Second, it is the use of 10 test guinea pigs and 5 controls that have been compared most frequently with the standard protocol.

The use of 5 rather than 10 control animals will have little influence on the accuracy of guinea pig tests. With respect to the number of test animals employed, it must be acknowledged that a reduction from 20 to 10 is associated with some potential reduction in overall accuracy. The reduction in accuracy will be most marked in guinea pig maximisation tests where a net response of approximately 30% is obtained (Shillaker *et al*, 1989); the estimation being that at most there will be a 12% change in sensitivity. However, it must be noted that surveys of guinea pig sensitisation testing reveal that only a small percentage of chemicals induce responses of approximately 30% (the minimum response necessary to classify a chemical as a skin sensitiser in the guinea pig maximisation test according to current EC criteria) and for this reason the overall reduction in accuracy when all test chemicals are considered is likely to be in the range of 1 to 2%. It should be noted also that rechallenge may be appropriate for borderline responses and this would also serve to compensate for any reduction in overall accuracy (Section 1.4). It is the view of this Task Force that the cost of this level of reduction in overall accuracy is more than compensated for by the animal welfare advantages that would result from the use of fewer guinea pigs. This view is supported by the analyses conducted by Hofmann *et al* (1987) who reported that the number of test animals could be reduced from 20 to 10 in the guinea pig maximisation test without compromising the utility of the assay.

While the available data suggest that there will be only a slight reduction in the accuracy of the guinea pig maximisation test with respect to classification and labelling, the situation for the occluded patch test (Buehler test) may be somewhat different. In this case a net response of 15%, rather than 30%, is used as the criterion for classification. Naturally, definition of a 15% net responses is less easy with the use of 10 test animals and 5 controls. Nevertheless, it may be that in certain circumstances the use of fewer animals could be accommodated here also.

Finally, it must be emphasised that the decision to reduce the number of test animals employed in guinea pig sensitisation assays must be based upon the needs and experience of individual laboratories and may not always be appropriate.

1.1.4 Benefits

A reduction where appropriate in the number of test and control animals will confer substantial animal welfare benefits.

1.2 Joint Positive Conrols

1.2.1 Background

The OECD Guideline 406 (1992) and the EC Test Method B6 (1996) require regular (every six months) confirmation of the reliability of the relevant guinea pig method and also the sensitivity of the strain of animals used. However, the location of the testing laboratory with responsibility for performing such positive control studies is not specified. Nevertheless, in practice, each laboratory performing guinea pig assays provides these data and is therefore required on an annual basis to conduct at least two positive control studies with specified contact allergens (see Section 1.3).

1.2.2 Recommendation

An optional scheme is proposed for the conduct of positive control studies within a co-ordinated group of laboratories, rather than by individual facilities. Specifically, the scheme would allow the group of laboratories to perform positive control studies on a regular basis, but with the responsibility for conducting such analyses being rotated within the co-ordinated group of laboratories.

1.2.3 Rationale

With the aim of reducing the number of animals required for routine reliability and sensitivity studies, an inter-laboratory collaboration was initiated recently in Germany under the auspices of the VCI (Verband der Chemischen Industrie) to consider whether the same studies could be conducted satisfactorily by a co-ordinated group of laboratories. From the experience gained it has been concluded that, if properly managed, a co-ordinated inter-laboratory approach to positive control testing is fully acceptable.

The basic requirements for the conduct of such joint positive control studies can be summarised as follows:

- A full and formal agreement on the management of the project group and the conduct and interpretation of studies should be in place.
- The project group should comprise a limited number (less than 10) of experienced laboratories.
- All participating laboratories must use guinea pigs of the same strain and deriving from the same breeder and supplier.
- All joint positive control studies conducted by the participating laboratories must be performed under GLP conditions.
- A single specified reference chemical allergen must be used for positive control studies by each of the participating laboratories (see Section 1.3).
- A common agreed detailed protocol for conduct of guinea pig studies must be used in each of the participating laboratories.
- A common and consistent approach to the evaluation of dermal responses and the interpretation of test data must be applied in all participating laboratories.

- There must be in place a system for continuing and regular cross-checks between laboratories.
- There must be access to positive control study reports by all participating laboratories.

To achieve the above there must be close and continuing liaison between the testing laboratories to ensure a consistency of approach and interpretation. Such consistency of evaluation and interpretation must first be achieved by close scientific collaboration and confirmation by joint assessments so that a uniform approach is adopted by each of the participating laboratories. Once a common procedure has been agreed, and a consistent approach to study conduct established, then it is possible for individual laboratories to perform (on a rotating basis) positive control studies on behalf of the consortium of participating laboratories.

1.2.4 Benefits

Adoption of this proposal would provide the following important benefits:

- A reduction in the number of guinea pigs required for reliability and sensitivity checks.
- An increase in efficiency of guinea pig sensitisation tests.

A further but indirect benefit is a more harmonised approach to the conduct of guinea pig tests and the interpretation of assay data.

1.3 *Positive Control Substance*

1.3.1 Background

In an update to Guideline 406, the OECD (1992) recommended the use of mildly/moderately sensitising positive control substances for the six-monthly assessment of the sensitivity and reliability of guinea pig tests. The suggested substances were hexyl cinnamic aldehyde (HCA; CAS no. 101-86-0), mercaptobenzothiazole (MBT; CAS no.149-30-4) and benzocaine (CAS no. 94-09-7). Only one of these three has to be selected for testing every six months.

Shortly thereafter, experience with the testing of each of these three positive controls in the OECD recommended guinea pig procedures was published (Basketter *et al*, 1993). Clear positive results were obtained only with HCA and MBT. Detailed experience with benzocaine demonstrated that results with this chemical were not reproducible, rendering it unsuitable for use as a positive control standard (Basketter *et al*, 1995).

In contrast, HCA has been shown to yield reproducible positive results in both OECD recommended protocols in a number of laboratories (Basketter *et al*, 1993; Basketter and Gerberick, 1996). Furthermore, the response to HCA has been demonstrated to be reproducible over a period of time (Basketter *et al*, 1999a). In addition to the published studies cited above this has been the experience of other company laboratories represented by the Task Force members.

1.3.2 Recommendation

Only one positive control substance (of the three possible chemicals identified currently by the OECD) is required for routine assessment of test sensitivity. The preferred chemical is hexyl cinnamic aldehyde (HCA).

1.3.3 Rationale

The majority of laboratories use either HCA or MBT as their choice of positive control, no doubt in part reflecting the published experience (Basketter *et al*, 1995). However, even this choice is not in fact necessary. The purpose of the positive control is to demonstrate the effectiveness of the protocol and the sensitivity of the strain. Since it is the technical aspects of test conduct that are being examined, and the mechanisms of skin sensitisation involved in guinea pig predictive testing are essentially the same for all chemicals, in practice only one appropriate positive control is required. The recommendation is that this material should be HCA. This chemical is a good choice for a positive control for the following reasons:

- HCA is readily available.
- HCA is free from other major toxicities (safe handling).
- HCA is not associated with any adverse reactions in the guinea pig, other than skin sensitisation.

- The response to HCA in properly conducted OECD guinea pig tests is reproducible, both in different laboratories and over time.
- Many laboratories already have a valuable background experience of using this substance as a positive control.
- HCA possesses a suitable degree of sensitising potential for the purpose, being neither too potent nor too weakly sensitising. As such it represents a reasonable test of the quality of the selected protocol and the sensitivity of the strain.

It is accepted that MBT also has some of the above characteristics, but there are fewer data available on reproducibility, and it is more strongly sensitising and malodorous.

1.3.4 Benefits

A single global positive control standard which would greatly facilitate inter-laboratory comparison of skin sensitisation test data.

1.4 Rechallenge in Guinea Pig Tests

1.4.1 Background

In guinea pig skin sensitisation testing, it is the response in the test group versus that in the control group (at 24 or 48 hours after the end of challenge) that determines whether the reactions in the test group should be interpreted as indicative of contact sensitisation. Guinea pig testing can be supplemented with a second challenge (a rechallenge). Rechallenge is considered to be a valuable tool as it may help:

- To evaluate questionable reactions obtained after initial challenge. For example, when it is unclear whether a response observed during the challenge phase is the result of primary irritation or is indicative of contact sensitisation, a rechallenge conducted after one to three weeks will improve the interpretation of test results if there is an altered irritation state of the skin.
- To clarify cross-challenge patterns of chemically related substances.
- To provide elicitation (challenge) dose-response information in the context of risk assessment.

It is recognised that in the current OECD 406 guideline (1992) the importance of a rechallenge is considered thus: "If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group."

1.4.2 Recommendation

The Task Force considers rechallenge an important tool for confirmation of the presence or absence of sensitisation. A proper rechallenge can provide information on persistence of the sensitisation response in individual animals, or in the test group as a whole.

1.4.3 Rationale

This section is a summary of relevant literature and the experience of Task Force members on the subject (Stotts, 1980; Robinson *et al*, 1989 and 1990; Kligman and Basketter, 1995; Frankild *et al*, 1996; Prinsen *et al*, 1997; Stropp *et al*, 1999) with an emphasis on when to perform a rechallenge and how to interpret the data.

A rechallenge is generally conducted in the same manner as the first challenge. The concentration chosen for rechallenge depends on the test reactions of the initial challenge and the concentration chosen to produce them. It should be recognised that the elicitation of an allergic patch test reaction is dose dependent. Selection of an inappropriately low challenge concentration may result in failure to elicit an allergic reaction in a sensitised animal, causing a "false-negative" patch test reaction. In contrast, application of too high a challenge concentration may cause "false-positive" irritant reactions.

It is important to conduct a rechallenge on all test animals and appropriate controls. Most rechallenge experiments are conducted one to three weeks after the initial challenge.

A. When to perform a rechallenge

Below are examples (derived from the experience of Task Force members) where rechallenge is appropriate in guinea pig testing.

- Any positive skin reaction that occurs in a test group in the absence of similar control group reactions, should in principle be interpreted as a possible indication of sensitisation. However, there is often a degree of uncertainty. At least one of the test group reactions should persist until the 48 hour time point and there should be a "clean" control group (i.e. no skin grades ≥ "1"). In general, a borderline incidence of positive reactions in the test groups (for instance with respect to EC labelling requirements) and / or positive reactions in both test and control groups or rapid fading of reactions after the first reading, would be considered questionable; in such cases a rechallenge is appropriate.
- The severity of positive skin reactions (although of secondary importance to incidence in data interpretation) can clearly aid in the interpretation of results or in determination of subsequent steps in the testing programme. Certainly the occurrence of grade "2" or "3" skin reactions is indicative of sensitisation. Even if grade "1" irritation reactions were observed in the control group, the higher test group reactions would be suggestive of an additive sensitisation response. This would guide the investigator to rechallenge at a lower concentration in order to eliminate the irritation reactions and determine whether any presumptive sensitisation reactions were maintained in the test animals. As a general rule, a two-fold reduction in concentration may be indicated where a low level irritation is suspected of complicating the reading.
- A significantly higher percentage of equivocal skin reactions in the test group versus the control group might indicate that the challenge concentration was too low. Follow up testing with a moderately increased concentration would be recommended since positive test group reactions might be expected to occur upon higher-dose rechallenge.

B. Vehicle considerations in rechallenge

Obviously, the ideal vehicle is one that solubilises or gives a stable suspension or emulsion of the test material and yet does not alter it, is free of allergenic potential, is non-irritating, enhances delivery across the stratum corneum, and reflects usage conditions of the test sample. However, in practice the choice of a vehicle is a compromise. In guinea pig testing, the same vehicle should normally be used for both induction and challenge. However, there are examples of ambiguous results when the same vehicle is used for induction and challenge. If equivocal results occur after challenge, the vehicle may be substituted for rechallenge to avoid non-specific hyperreactivity. If there is a potential for vehicle sensitisation, then the test and control animals should receive an additional patch of the vehicle alone at challenge and rechallenge. Finally, rechallenge can in theory also be used to evaluate the effect of various vehicles on the intensity of the sensitisation response.

C. Data interpretation in rechallenge experiments

The same criteria used for interpreting challenge reactions should also govern interpretation of rechallenge reactions. However, there is a need to adopt a holistic approach to evaluating the entire study. For example, if positive results were observed at challenge at a given concentration, then questionable or negative results at the same concentration at rechallenge would render the entire study questionable or negative. However, questionable or negative rechallenge results at a lower concentration would not affect the interpretation since such a dose response would be expected. In contrast, equivocal challenge data followed by positive rechallenge data, at any concentration, would render the entire study positive.

Challenge reactions, even weak ones, that are truly allergic in nature can generally be reproduced over a period of at least several weeks. Non-specific irritation reactions, even strong ones, diminish or disappear on rechallenge within two to three weeks; in fact, weak irritant reactions may not be repeatable after one week. In contrast, moderately strong allergic reactions can be evoked at nearly the same intensity for periods of two to three months. After five to six months, the animals generally show weaker allergic reactions, although complete loss of the allergic state is uncommon.

It must be emphasised that grade “1” skin reactions may be truly allergic. In highly sensitised animals and humans, it is always possible to dilute the allergen to a concentration that provides only grade “1” reactions. These are indistinguishable from purely irritant reactions. Often these will still be grade “1” at 48 hours, while irritant reactions will generally have faded by that time. It should also be borne in mind that allergenicity is a delicate balance between immunoregulatory mechanisms, and that the substance tested may influence this balance as a consequence of the first challenge procedure. Ultimately, any rechallenge data must be interpreted with care in the context of the entire study. Interpretation has to be conducted on a case-by-case basis, using as a guide the best understanding of immunology integrated with experience of test conduct (see below).

D. Worked examples

Table 1: Examples of rechallenge demonstrating a weak sensitisation reaction

	Challenge results †			
	Challenge 1		Rechallenge	
Animal Number	**24h**	**48h**	**24h**	**48h**
test 1	0	0	0	0
test 2	0	1	1	1
test 3	0	1	1	2
test 4	0	0	0	0
test 5	0	0	0	0
test 6	0	0	0	0
test 7	1	1	1	1
test 8	0	0	1	1
test 9	0	1	0	0
test 10	0	0	0	0
control 11	1	0	0	0
control 12	0	0	0	0
control 13	0	0	0	0
control 14	0	0	0	0
control 15	0	0	0	0

† Expressed as erythema on a scale of 0-3 and scored 24h and 48h after removal of patch: A "2" reaction is moderate erythema, "1" is weak, usually homogenous, erythema. Rechallenge was conducted one week after Challenge 1

In Table 1 it can be seen that there are some low grade reactions after the first challenge, particularly at the later scoring time point. However, these are no greater than the single irritant reaction noted in a control animal. The tendency of most of the responses to occur at the later time point implies they are more likely to be skin sensitisation than irritation. Rechallenge under identical conditions on the opposite flank demonstrates that the majority of the reactions in the test guinea pigs are reproducible, they have still a tendency to be more pronounced at the later time point and have increased slightly; the absence of a response in control animals further confirms the allergic nature of the reactions.

Table 2: Examples of rechallenge demonstrating non-allergic reactions

	Challenge results †			
	Challenge 1		Re-challenge	
Animal Number	**24h**	**48h**	**24h**	**48h**
test 1	0	0	0	0
test 2	2	1	0	0
test 3	1	1	0	0
test 4	0	0	1	0
test 5	0	0	0	0
test 6	0	0	0	0
test 7	0	0	0	0
test 8	0	0	0	0
test 9	1	0	1	0
test 10	0	0	0	0
control 11	0	0	0	0
control 12	0	0	0	0
control 13	0	0	0	0
control 14	0	0	0	0
control 15	0	0	0	0

† Expressed as erythema on a scale of 0-3 and scored 24h and 48h after removal of patch. A "2" reaction is moderate erythema, "1" is weak, usually homogenous, erythema. Rechallenge was conducted one week after Challenge 1

In Table 2, the initial challenge indicated that three guinea pigs had been sensitised. However, the nature of the reactions (fading at the later time point in two cases) suggests they may in fact be due to skin irritation. Rechallenge under identical conditions on the opposite flank shows that the responses are not reproducible in the guinea pigs reacting at challenge 1, notably in the strongest reacting animal and overall there is a reduced level of response. Furthermore, they continue to demonstrate fading at the later scoring time. Thus, despite any evidence of irritation in controls, the reactions in the test animals are not of an allergic nature.

1.4.4 Benefits

The results of a properly performed rechallenge, in the context of the results from the entire study, should avoid misinterpretation of guinea pig skin sensitisation studies and the need for unnecessary repeat investigations.

1.5 SLS Pretreatment

1.5.1 Background

The current OECD guideline for the guinea pig maximisation test (1992), requires treatment of the test site with SLS 24 hours prior to application of a non-irritant test substance. This requirement is based on the studies of Magnusson and Kligman (1969) who proposed the use of SLS to provoke a mild to moderate inflammatory reaction at the application site.

1.5.2 Recommendation

The Task Force recommends that sodium lauryl sulphate (SLS) is no longer used as a pretreatment in the guinea pig maximisation test.

1.5.3 Rationale

The rationale for this recommendation was that pretreatment with SLS would facilitate the percutaneous absorption of the test material. It is now considered that such treatment is unnecessary and in the interests of both good scientific practice and animal welfare considerations should be discontinued for the following reasons:

- In the guinea pig maximisation test Freund's Complete Adjuvant (FCA) is given by intracutaneous injection to enhance immune responses. This is administered at the application site and as a consequence the skin is already inflamed even in the absence of SLS.
- The rationale for the use of SLS is based upon the assumption that such treatment will facilitate the absorption of the test material. However, the critical event in this context is the ability of the test chemical to gain access to the viable epidermis where interaction with Langerhans cells takes place and the induced or increased production of relevant skin cytokines is stimulated. The available data suggest that topical treatment with SLS can enhance the systemic absorption of chemicals without increasing the amount of material found within the viable epidermis. Thus, there is no clear evidence that SLS routinely promotes the entry of chemical into the viable epidermis. On the contrary, in some instances the concentration of test chemical within the epidermis has been shown to be reduced following pretreatment with SLS (Wilhelm *et al*, 1991; Maurer, 1996). Further, although the notion is that SLS pretreatment may be effective in enhancing skin reactions in guinea pigs to weak contact allergens, the basis for such observations is not clear (Prinsen *et al*, 1997 and 1999), and not consistent with the experience of other investigators (Stropp *et al*, 1999).
- Pretreatment with SLS may compromise the scientific integrity of the test as it may result in hyperirritable skin, a decrease in the irritation threshold and an associated risk of “false positive” reactions (Kligman and Basketter, 1995; Buehler, 1996; Middleton

et al, 1998; Stropp, 1998a). In humans also, treatment with SLS has been associated with false positive skin reactions (Kligman and Epstein, 1975). Conversely, there is some evidence that SLS may have the potential to suppress skin reactivity with the risk of "false-negative" results (Bruynzeel *et al*, 1983; Jokipii and Jokipii, 1973; McGuire and Fox, 1979; Uehara and Ofuji, 1977). A non-specific hypersensitivity induced by pretreatment with SLS may cause lowered or "false-negative" responses in cases where a reduction in irritation threshold has influenced the challenge concentration based on range-finding studies conducted with SLS-treated guinea pigs (Stropp, 1998b and c).

- There is limited evidence to suggest that in some circumstances SLS may itself act as an allergen (Sams and Smith, 1957; Prater *et al*, 1978; Fisher, 1995; Basketter *et al*, 1996a).
- The guinea pig maximisation test requires an aggressive induction regime that even in the absence of SLS pretreatment is traumatic and characterised by marked skin inflammation resulting from treatment with FCA. Further irritation resulting from exposure to SLS may compromise performance of the test while adding significantly to the trauma to which guinea pigs are potentially subject.

1.5.4 Benefits

Discontinuation of the use of SLS as a pretreatment in the guinea pig maximisation test will:

- Improve the performance of the assay.
- Reduce the incidence of "false positive" and (in certain instances) "false negative" reactions.
- Reduce the trauma to which animals are subject; an important refinement in the context of animal welfare considerations.

2. MURINE LOCAL LYMPH NODE ASSAY

2.1 Background

More than ten years ago the local lymph node assay (LLNA) was described (Kimber *et al*, 1986; Kimber *et al*, 1989), a standard protocol then prepared (Kimber and Basketter, 1992) and subsequently the data produced were reviewed (Kimber *et al*, 1994; Kimber, 1996). This method was founded on the understanding that an increasingly sophisticated appreciation of the immune system would facilitate the design of alternative methods for the identification of contact allergens. The LLNA employs mice, the experimental species in which the most detailed information about the induction and regulation of immunological responses is available. In contrast to guinea pig test methods, the LLNA identifies potential skin sensitising chemicals as a function of events associated with the induction, rather than elicitation, phase of skin sensitisation. The induction phase of skin sensitisation is characterised by the stimulation of an allergen-specific immune response in lymph nodes draining the site of exposure. The importance of the clonal expansion of T lymphocytes is reflected by the fact that the vigour of proliferative responses induced by chemical allergens in draining lymph nodes correlates closely with the extent to which sensitisation will develop (Kimber and Dearman, 1991 and 1996). It is upon measurement of this response that the LLNA is based.

2.2 Recommendation

The LLNA is a viable and complete alternative to traditional guinea pig methods for the purposes of hazard identification. The LLNA offers a substantial reduction in animal numbers and refinement opportunities without compromising the standards for the identification of skin sensitisers.

2.3 Rationale

The LLNA has been the subject of both national (Basketter *et al*, 1991; Kimber *et al*, 1991; Scholes *et al*, 1992) and international (Kimber *et al*, 1995; Loveless *et al*, 1996; Kimber *et al*, 1998) collaborative trials and of rigorous comparisons with guinea pig tests and human sensitisation data. The overall conclusion from these validation studies is that independent laboratories, despite the use of minor procedural modifications and different methods for data analysis, successfully and consistently reached identical conclusions regarding the sensitising potential of 40 different chemicals, using the LLNA.

On the basis of these investigations, the LLNA has been considered recently by the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) which concluded that the method, in modified forms, is sufficiently validated as a stand-alone test for the identification of skin sensitising chemicals. In addition, the ICCVAM peer review panel confirmed that the LLNA offers important animal welfare benefits by refining the way in which animals are used for skin sensitisation testing and reducing the number of animals required for this purpose (ICCVAM, 1999; Gerberick *et al*, 2000).

In conclusion the LLNA, or modified versions of this test, provide a viable alternative method for use in the identification of skin sensitising chemicals and for confirming that chemicals lack a significant potential to cause skin sensitisation. This does not necessarily imply that in all instances the LLNA should be used in place of guinea pig tests, but rather that the assay is of equal merit and utility and may be employed as a full alternative in which positive and negative results require no further confirmation.

2.4 Benefits

The LLNA is not an *in vitro* method and as a consequence will not eliminate the use of animals in the assessment of contact sensitising activity. It will, however, permit a reduction in the number of animals required for this purpose. For each chemical tested, the number of animals required for a LLNA is, on average, half that needed for a standard guinea pig test. Moreover, the LLNA offers a substantial refinement of the way in which animals are used for contact sensitisation testing. One important point is that, unlike some of the guinea pig methods, such as the guinea pig maximisation test, the LLNA does not require the use of adjuvant. Furthermore, the LLNA is based upon consideration of immunobiological events stimulated by chemicals during the induction phase of sensitisation. Therefore, unlike guinea pig tests the LLNA eliminates the need for challenge-induced dermal hypersensitivity reactions. Associated with this is the fact that, unlike guinea pig tests, the performance of the LLNA is not compromised when coloured chemicals are tested, which in guinea pigs can stain the challenge site. Further, the time taken for conduct of an LLNA is considerably less than that required for a standard guinea pig method.

CONCLUSIONS

The conduct of guinea pigs tests and of the murine local lymph node assay have been considered in the context of skin sensitisation hazard identification, and the potential for animal welfare benefits.

With respect to guinea pig tests, recommendations are made to enhance the performance of these methods while providing a number of important animal welfare benefits.

In addition, the murine local lymph node assay is endorsed as a stand-alone alternative to standard guinea pig tests. This method confers a number of advantages, among these being significant animal welfare benefits in terms of reduction and refinement.

BENEFITS OF THE RECOMMENDATIONS

In summary, the major benefits of the recommendations made here are:

Animal Welfare

- Reduction in number of animals.
- Avoidance of unnecessary repeat investigations.
- Reduction in trauma to which animals are potentially subject.

Enhanced Performance/Acceptance

- Harmonised approach of conduct and interpretation of skin sensitisation testing.
- Interlaboratory comparisons.
- Avoidance of misinterpretation (rechallenge).
- Reduction in the incidence of “false positive” and “false negative” results.

PART TWO: RELATIVE POTENCY AND RISK ASSESSMENT

Introduction

In this second part of the Monograph, skin sensitisation test methods are considered in the context of their ability to provide estimates of relative potency and to contribute to the risk assessment/risk management process. The first step in any toxicological evaluation is the accurate identification of hazard. However, for an effective assessment of risk some knowledge of relative potency is necessary. This certainly applies to contact allergy where there is evidence that the relative skin sensitising potency of chemicals may vary by many orders of magnitude. We consider here the application of guinea pig tests (the guinea pig maximisation test, the occluded patch test and the open epicutaneous test), a mouse test (the local lymph node assay) and human sensitisation testing to determine relative skin sensitising potency and/or for the purpose of risk assessment. The remit addressed by the Task Force in this part was:

Review relevant skin sensitisation test methods and:

Make recommendations for the use of relevant skin sensitisation test methods for the purposes of (a) determination of relative potency and the threshold dose necessary for the induction of skin sensitisation and (b) risk assessment.

This remit is addressed under six headings:

- Dose responses, thresholds and potency in skin sensitisation - general considerations.
- Guinea pig tests.
- Local lymph node assay.
- Human testing.
- Application of test methods in risk assessment.
- General conclusions and recommendations.

1. DOSE RESPONSES, THRESHOLDS AND POTENCY IN SKIN SENSITISATION - GENERAL CONSIDERATIONS

Skin sensitisation describes an immunological process whereby heightened responsiveness to a chemical allergen is induced. In addressing questions of dose responses, thresholds and potency in skin sensitisation it is necessary first to consider in general terms the immunobiological processes that result in the acquisition of skin sensitisation and the elicitation of contact dermatitis.

By definition skin sensitisation is induced when a susceptible individual is exposed topically to the inducing chemical allergen. This chemical allergen provokes a cutaneous immune response which, if of the required magnitude and quality, will result in the development of contact sensitisation. A number of key steps in this process can be identified.

To elicit an immune response a chemical must gain access to the viable epidermis. As a consequence a skin sensitising chemical requires the physicochemical characteristics necessary for passage across the *stratum corneum*, which under normal circumstances represents an effective barrier to many chemicals. In their native state chemical allergens are haptens and as such are of insufficient size to provoke an immune response. For this to be achieved the chemical must form stable conjugates with macromolecular proteins. As a consequence skin sensitising agents are either inherently protein-reactive or can be metabolised in the skin to a protein-reactive species (Barratt and Basketter, 1992; Basketter, 1992; Roberts and Lepoittevin, 1997). Absorption of the allergen into the skin is important and dependent on several factors, some of which have been modelled mathematically (Roberts and Williams, 1982). What is required is for the reactive chemical moiety to persist for sufficient time and in sufficient amounts at the target site, the viable epidermis.

In the epidermis there exists an interdigitating network of Langerhans cells (LC). These cells form part of a wider family of dendritic cells (DC), the main function of which is to present antigen to the immune system. Following skin exposure, epidermal LC internalise and process the inducing allergen. A proportion of the LC local to the site of exposure (some of which bear high levels of antigen) is stimulated to migrate from the epidermis and to travel via afferent lymphatics to skin draining lymph nodes. The cells that move from the skin are subject to a functional maturation such that they accumulate in draining lymph nodes as immunostimulatory DC which are able to present antigen effectively to responsive T lymphocytes (Kimber and Cumberbatch, 1992). The antigen-driven activation of T lymphocytes is characterised by cell division and differentiation. The proliferation of allergen-responsive T lymphocytes results in clonal expansion, in effect increasing the complement of cells that are able to recognise and respond subsequently to that same inducing allergen. It is this selective clonal expansion of T lymphocytes that provides immunological memory and that represents the cellular basis for skin sensitisation. If the now sensitised subject is exposed again to the inducing chemical allergen, at the same or a different site, then this expanded population of specific T lymphocytes will recognise and respond to the allergen in the skin at the site of contact. The activation of T lymphocytes is associated with the release of cytokines and

chemokines that collectively stimulate the influx of other leukocytes and initiate the cutaneous inflammatory reaction that is recognised clinically as allergic contact dermatitis (Kimber and Dearman, 1996).

The evidence available from animal studies indicates that the above processes display dose response relationships, which in turn are associated with the effectiveness of sensitisation (Kimber and Basketter, 1997). In general terms, the greater the level of exposure to allergen, the more vigorous will be the induced immune response and the greater the level of sensitisation achieved. If one considers separately the individual processes that together result in sensitisation then the following relationships can be discerned. First, the stimulation of LC migration and DC accumulation in draining lymph nodes are dose-dependent phenomena (Kinnaird *et al*, 1989). In addition, the more effective the accumulation of DC in draining lymph nodes, the more vigorous the proliferative response by lymph node cells (Kimber *et al*, 1990). Finally, the effectiveness of skin sensitisation has been shown in mice to correlate with the magnitude of allergen-induced lymph node cell proliferative responses (Kimber and Dearman, 1991). In summary, the cellular and molecular processes that are initiated in skin and skin-associated lymphoid tissue following topical exposure to a sensitising chemical culminate in the induction of a T lymphocyte response. If this is of sufficient vigour (and subject to inherent homeostatic immuno-regulatory mechanisms), acquisition of skin sensitisation will result.

On the basis of these considerations it is possible to draw certain inferences about the nature of dose responses, thresholds and potency in the context of experimental skin sensitisation. It is clear that in mice, guinea pigs and humans, the induction of skin sensitisation and the elicitation of allergic contact hypersensitivity reactions are dose-dependent phenomena; in both instances it is possible to determine threshold concentrations of allergen required for a response or reaction to be initiated (Kimber *et al*, 1999). With respect to the induction of sensitisation in a previously naïve animal the threshold can be regarded as the concentration of chemical allergen necessary to provoke a cutaneous immune response of sufficient magnitude to result in sensitisation. At the elicitation stage, a threshold value is best defined as that challenge concentration of the inducing allergen that is required to elicit a detectable cutaneous hypersensitivity reaction in a previously sensitised animal (Kimber *et al*, 1999).

The potency of a chemical contact allergen should be defined in terms of such threshold values. This applies to both the induction and elicitation phases of skin sensitisation, although the more important consideration is the relative ability of chemical allergens to cause sensitisation in a previously unsensitised (but nevertheless inherently susceptible) subject. If the relative potency of allergens is described in terms of the amount of chemical necessary to induce sensitisation (or a defined response linked causally and quantitatively with the acquisition of sensitisation), then it follows that for weaker allergens, sensitisation will require topical exposure to larger amounts than is necessary for sensitisation to stronger allergens. It is important to emphasise this point because there often exists confusion between the true intrinsic potency of a chemical allergen and the prevalence of sensitisation among human populations or the frequency of positive responders in

guinea pig predictive tests. In the case of human allergic contact dermatitis the prevalence of sensitisation to any one chemical is as much a function of opportunities for exposure (and, in particular, the nature, extent and duration of skin contact) as it is the inherent potency of the chemical allergen. Thus, for instance, although nickel is the most common cause of skin sensitisation in Western Europe, it is not a strong allergen and in fact has relatively weak sensitising potential. The high prevalence of nickel allergy results instead from the widespread opportunities for exposure to this metal (Kimber and Basketter, 1997).

In summary, the evidence available from experimental investigations reveals that skin sensitisation, and the elicitation of allergic contact hypersensitivity reactions, are dose-dependent responses for which threshold values can be identified for particular exposure conditions. Potency is best defined as the amount of test chemical required to cause sensitisation or to induce a certain magnitude of response linked causally and quantitatively with the acquisition of skin sensitisation.

It is relevant now to consider what is known of dose responses, thresholds and potency from clinical research studies. Some of the most informative investigations were described by Friedmann and colleagues who examined skin sensitisation in human volunteers to the potent contact allergen 2,4-dinitrochlorobenzene (DNCB) (Friedmann, 1990 and 1996). Several important points can be made from these studies.

One of these is that in humans the incidence of skin sensitisation is dose dependent. Previously unsensitised volunteers were exposed topically to various amounts of DNCB and the incidence of sensitisation assessed subsequently as a function of challenge-induced cutaneous hypersensitivity reactions. It was found that the proportion of subjects sensitised displayed a positive sigmoid relationship with the log of the sensitising dose (Friedmann and Moss, 1985).

Perhaps the most seminal observation made was that under normal circumstances the effectiveness of skin sensitisation is dependent upon the amount of chemical allergen per unit area of skin (rather than the total amount delivered). When increasing total amounts of DNCB were applied to proportionally larger areas of skin it was found that the percentage of sensitised subjects remained unchanged, this being despite the fact that groups of volunteers had received exposure to very different total doses of the allergen. Associated with this was the finding that when the total amount of allergen remained constant, but the area of skin over which it was applied changed, then the incidence of sensitisation was greater in groups that had received the standard amount of DNCB over a smaller area (White *et al*, 1986). This relationship between the effectiveness of sensitisation and the amount of chemical encountered per unit area of skin holds true for most normal conditions of exposure where the application site is greater than a critical minimum area. Rees *et al* (1990) showed that when exposure sites of less than 1 cm^2 were used the area over which the chemical was applied became a critical determinant of the acquisition of sensitisation. The implication is that in most cases it is the amount of chemical per unit area of skin, rather than the total level of exposure, that is the most important variable in effecting sensitisation. This is because a certain level of

allergen-induced changes is required locally to provoke a cutaneous immune response of sufficient vigour. However, when the area becomes very small then the relationship breaks down because in such circumstances the availability of certain critical elements in the skin becomes limiting. It could be argued, for instance, that where the application site is very small there will be insufficient epidermal Langerhans cells to support the development of a cutaneous immune response, irrespective of the local concentration of chemical allergen.

In conclusion, it is possible based upon the available clinical and experimental data, to define the following characteristics of skin sensitisation:

- The induction of skin sensitisation in a naïve individual or animal and the elicitation of a cutaneous allergic hypersensitivity reaction in a previously sensitised subject or animal are dose-dependent phenomena. In both cases threshold concentrations of chemical allergen can be defined below which the induction of sensitisation or the elicitation of a cutaneous allergic reaction will fail to develop.
- Under normal conditions of exposure it is the amount of chemical allergen per unit area of skin (rather than the total amount delivered) that is the most important determinant of the acquisition of sensitisation.
- It is likely that the elicitation of allergic contact dermatitis in a previously sensitised subject is similarly influenced by the amount of chemical allergen encountered per unit area of skin, but this has not been formally proven.
- The potency of a chemical allergen is of greatest significance when determining the risk of inducing skin sensitisation in a previously unsensitised individual. Potency is considered here in terms of the amount of chemical (per unit area of skin) that is required to induce sensitisation or to provoke a certain vigour of response that is linked causally and quantitatively to the induction of skin sensitisation.

2. GUINEA PIG TESTS

2.1 Background

Guinea pig tests, in particular the two methods recommended in the OECD and EC test-guidelines for skin sensitisation testing, the guinea pig maximisation test (GPMT) (Magnusson and Kligman, 1970) and the occluded patch test of Buehler (Buehler, 1965) have been used extensively for identification of skin sensitisation hazards (Andersen and Maibach, 1985; Botham *et al*, 1991a; Kligman and Basketter, 1995; Basketter *et al*, 1999a). The purpose of these tests is to identify skin sensitisers effectively. This compromises to some extent their ability to provide information on relative sensitising potency, due to the unusual routes of exposure, choice of vehicle, use of adjuvant and selection of test substance concentrations based on skin irritancy and/or toxicity. However, in many instances the guinea pig tests represent the only available database on the skin sensitisation potential and potency of chemicals. In addition to these two standard methods, other approaches using guinea pigs, such as the open epicutaneous test (OET), offer some opportunity to evaluate relative skin sensitising potency in greater detail. In this section the use of such methods for the assessment of relative potency and their advantages and limitations in the context of risk assessment are discussed. Examples are also given.

2.2 Guinea Pig Tests and Sensitisation Potency Assessment

When attempting to determine skin sensitising potency based on the results of guinea pig tests several variables should be considered. These include:

- concentration(s) applied during the induction phase;
- vehicle(s) used during the induction phase;
- concentration(s) applied during the challenge phase;
- vehicle used during the challenge phase;
- impact of the relative contribution of epicutaneous versus intradermal routes of exposure (GPMT);
- proportion of guinea pigs with positive challenge reactions;
- intensity of reactions;
- kinetics of responses;
- dose-response curve (if available).

Guinea pig tests, particularly those involving the use of adjuvant, may occasionally present difficulties of interpretation arising from the irritancy of the test substance (Kligman and Basketter, 1995; Buehler, 1996). One additional point is that applications are frequently dictated by skin irritant activity.

Strategies to distinguish such "false positives" from true sensitisers are now well described (Kligman and Basketter, 1995; Frankild *et al*,1996; Basketter *et al*, 1998). It is worth considering what has been achieved using the guinea pig tests with regard to estimation of relative skin sensitising potency.

2.2.1 Guinea pig maximisation and Buehler tests

With the earliest publications of the GPMT (Magnusson and Kligman, 1969 and 1970) it was evident that the most potent sensitising chemicals, such as DNCB and p-phenylenediamine, sensitised a higher proportion of the test animals than did weaker sensitisers such as nickel sulphate or benzocaine. A similar pattern was found with the Buehler test, although published information has been until recently in short supply (Basketter and Gerberick, 1996).

Typically, the emphasis in considerations of potency has been on the number of animals that display positive reactions following challenge. Nevertheless, there may be substantial differences in the potency of an allergen which produces a 100% rate of sensitisation at very low concentrations, compared with another which requires very high test concentrations to achieve the same result (Roberts and Basketter, 1990). However, the mathematical relationships for establishing relative potency between such allergens are complex, not least because the relative contributions of the induction and elicitation concentrations to the overall result are unclear. Indeed, such relationships may vary between different chemical sensitisers.

Andersen and co-workers have attempted to modify the GPMT to provide information on sensitising potency (Andersen *et al*, 1995). Their approach involves the use of multiple dose levels at the induction stage. However, this modified protocol has been evaluated with only a few skin sensitisers, and the data generated suggest that the approach is not of great value for the assessment of relative sensitising potency. A different strategy involving the definition of various standard doses has been proposed by others (Nakamura *et al*, 1994; Momma *et al*, 1998), but the complex scheme proposed and evaluated with only eight chemicals has not been endorsed by other investigators.

Generally, results from a Buehler test provide more relevant information with regard to threshold levels than do those deriving from methods that require intradermal application and/or FCA. Important reasons are that the route of exposure does not bypass the relevant skin barrier and that no additional stimulus (adjuvant) is used that will alter immune reactivity (Basketter *et al*, 1992).

2.2.2 Open epicutaneous test

When investigating relative potency, dose response and thresholds, it is relevant to consider one other guinea pig test, the OET (Klecak *et al*, 1977; Kero and Hannuksela, 1980; Anderson and Hamann, 1984; Klecak, 1985). The OET uses a multiple dose regime for induction and challenge that enables the determination of dose responses and thresholds for both phases. Groups of eight animals are exposed epicutaneously (without occlusion) using a single induction concentration (20 induction applications, five applications per week). Usually four test groups are incorporated in each study, each receiving different induction concentrations. All test animals, as well as corresponding controls, are treated identically at challenge using four concentrations applied simultaneously at four different skin sites. The highest concentrations may be at mildly irritant levels. Skin sensitisation is then determined by differences in the reaction threshold in test animals compared with controls for each induction and challenge concentration.

2.3 Examples

Provided below are examples of how guinea pig tests have been used to assess sensitisation potency.

Example No. 1

An interpretation of typical results in an OET (Table 3) is given below:

The highest challenge concentration chosen (5%) was irritant, as revealed by positive challenge results obtained with the control group. The threshold for induction of skin sensitisation in this test was found to be between 0.2% and 0.75%. No sensitisation was induced with 0.2%. The challenge threshold was dependent on the induction concentration used. When 5% was used for induction, no threshold for challenge could be determined with the concentrations tested. However, the declining frequency of positive animals indicated that a threshold would be achieved if lower challenge concentrations had been used (Table 3).

Table 3: Results of an OET

		Percentage of animals displaying positive reactions			
Dose group	**Dermal induction (% w/v)**	**Challenge (% w/v)**			
		5	2	0.75	0.2
Control group	0 (vehicle)	25	0	0	0
Test group 1	5	100	87.5	50	25
Test group 2	2	100	50	12.5	0
Test group 3	0.75	100	50	0	0
Test group 4	0.2	25	0	0	0

Example No. 2

With existing chemicals it is often the case that more than one assay will have been performed with the same material. Comparisons can then be made between test methods with regard to the value of the potency information that they provide.

The results of two independent GPMT studies (Tables 4 and 5) suggest some apparent potential for skin sensitisation. In contrast, however, results obtained from the OET (Table 6) indicate a lack of skin sensitising activity - even at the highest induction concentration of 30%. The human repeat insult patch test (HRIPT) confirms that under the conditions employed (an induction concentration of 20%), the test substance fails to induce sensitisation (Table 7).

Table 4: Results of the first GPMT with substance X

Intradermal induction (% w/v)	Dermal induction (% w/v)	Challenge (% w/v)	No. of animals	No. of animals displaying positive reactions
25	25	25/12.5	15	14/4
1	1	50/25	15	4/0

Table 5: Results of the second GPMT with substance X

Intradermal induction (% w/v)	Dermal induction (% w/v)	Challenge (%w/v)	No. of animals	No. of animals displaying positive reactions
5	10	1/0.5/0.1	19	16/15/12
1	10	1/0.5/0.1	20	10/15/12
1	10	1/0.5/0.1	20	14/13/10
0.2	10	1/0.5/0.1	20	12/15/9
0.2	2	1/0.5/0.1	20	1/2/1
0.2	0.48	1/0.5/0.1	20	6/6/4

Table 6: Results of the OET with substance X

Dermal induction (% w/v)	Challenge (% w/v)	No. of animals	No. of animals displaying positive reactions
30	100 30/10/3	7	slight irritation 0/0/0
10	100 30/10/3	7	slight irritation 0/0/0
3	100 30/10/3	7	slight irritation 0/0/0
1	100 30/10/3	7	slight irritation 0/0/0
0	100 30/10/3	7	slight irritation 0/0/0

Table 7: Results of the HRIPT with substance X

Dermal induction (% w/v)	Challenge (% w/v)	No. of subjects	No. of subjects displaying positive reactions
20	5	66	0
10	5	88	0
5	5	129	0

Controversial data from studies such as those outlined above provide information which must then be interpreted in the context of data available for other sensitising chemicals. Knowledge of the dose response for benchmark chemicals, in combination with information on the extent to which they cause human sensitisation resulting from particular types of exposure, enables a safety assessment to be made.

2.4 Advantages and Limitations

When evaluating the relevance of guinea pig tests for potency assessment, it is necessary to distinguish clearly between those methods that use adjuvant and/or intradermal administration and non-adjuvant tests. The interpretation of data from tests which employ adjuvant is complex and the results are not directly applicable to normal human use conditions. The approach of comparative interpretation of different tests performed with the same chemical together with the use of data for known benchmark chemicals can sometimes provide a suitable tool for evaluation of relative skin sensitising potency.

Advantages:

- Guinea pig test data for many existing chemicals are readily available and these may allow comparative interpretation. In such instances additional testing may not be necessary.
- Methods such as the OET provide dose-response information for the induction, as well as for the challenge, phase.
- In the OET, determination of sensitisation and elicitation thresholds is possible.

Limitations:

- The standard GPMT and Buehler tests performed with one induction concentration and one challenge concentration do not provide information on thresholds. (When more than one challenge concentration is introduced some information on dose-response may be obtained).
- The range for dose selection is limited and based on irritation threshold.
- Adjuvant tests are based on the principle of worst-condition testing and are therefore not suitable for potency assessment.
- For new chemicals normally only one study is likely to be available.

3. LOCAL LYMPH NODE ASSAY

3.1 Background

The murine local lymph node assay (LLNA) is a novel predictive method for the identification of skin sensitising chemicals where, in contrast to guinea pig tests, activity is judged as a function of the induction, rather than of the elicitation, phase of sensitisation (Kimber and Basketter, 1992; Kimber *et al*, 1994). Specifically, the LLNA measures the ability of topically applied chemicals to induce in mice proliferative responses by draining lymph node cells (LNC) (Kimber *et al*, 1994).

Before considering the potential utility of the LLNA for the purposes of determining relative potency, it is necessary to review briefly the relevance of the readout of this assay for skin sensitisation. The effective induction of contact sensitisation demands that chemical allergens encountered on the skin provoke a cutaneous immune response of sufficient vigour and of the appropriate quality. Skin sensitisation, and the allergic contact hypersensitivity reaction that is elicited following subsequent challenge, are T lymphocyte-dependent cellular immune responses. A defining event during the induction phase of skin sensitisation is the stimulation within draining lymph nodes of proliferative responses by allergen-reactive T lymphocytes. This in turn results in selective clonal expansion and an increased frequency of T lymphocytes that are able to recognise and respond to the inducing allergen. Expansion of allergen-responsive T lymphocytes represents the cellular basis of immunological memory and of skin sensitisation. If their function is impaired, or the stimulation of proliferative responses in draining lymph nodes inhibited, skin sensitisation is compromised or fails to develop (Kimber, 1989; Kimber and Dearman, 1997). It is therefore appropriate to consider the stimulation by topically applied chemicals of LNC proliferation in draining lymph nodes to be a relevant marker for the identification of skin sensitising chemicals.

However, it has been possible to demonstrate also that, consistent with the development of sensitisation being dependent upon T lymphocyte division, the vigour of induced LNC proliferative responses correlates closely with the extent to which contact sensitisation will develop (Kimber and Dearman, 1991).

3.2 The LLNA and Potency Assessment of Chemicals and Formulations

The quantitative association between LNC proliferative activity and skin sensitisation potential suggested that the LLNA might permit not only the accurate identification of contact allergens, but also assessment of relative sensitising potency (Kimber and Basketter, 1997). In accordance with general considerations of potency and thresholds in contact sensitisation (Part Two, Section 1, page 23) the strategy adopted was to define potency from LLNA responses as a function of the amount of test chemical required to elicit a predetermined level of LNC proliferative activity. The level chosen for this purpose was a 3-fold increase in proliferation compared with concurrent vehicle-treated controls and the concentration of chemical necessary to provoke this level of response was accordingly designated the EC3 value (Effective Concentration for a stimulation index of 3).

It is worth considering briefly the rationale for selection of the EC3 for this purpose. Early experience with the LLNA indicated that a stimulation index of 3 provided a reliable (although nevertheless empirical) discriminant between sensitising and non-sensitising chemicals. For this reason skin sensitising chemicals were defined as those which, at one or more test concentrations, elicited a 3-fold or greater increase in LNC proliferative activity compared with concurrent vehicle-treated controls (Kimber and Basketter, 1992). Although empirical, this criterion for a positive response has stood the test of time. Recent analyses employing a rigorous statistical evaluation of existing data have confirmed that a 3-fold increase in activity in the LLNA represents the most appropriate criterion for classification of chemicals as potential human contact allergens (Basketter *et al*, 1999b). It should be noted here that, as with guinea pig tests, certain strong irritants may give rise to "false positive" results in the LLNA. This issue, and the scientific interpretation of such results, have been reviewed elsewhere (Basketter *et al*, 1998).

In recent years derived EC3 values have been employed successfully for comparing the performance and sensitivity of the LLNA when the test is performed independently in separate laboratories with the same chemicals (Kimber *et al*, 1995; Loveless *et al*, 1996). More recently EC3 values have been applied to direct determination of the relative skin sensitising potency of chemicals. Included among the specific investigations conducted to date are, an evaluation of the relative sensitising potency of four dinitrohalobenzenes (Basketter *et al*, 1997a), a comparison of glutaraldehyde with formaldehyde (Hilton *et al*, 1998) and a comparative evaluation of three isothiazolinone biocides (Basketter *et al*, 1999c). Moreover, derived EC3 values have been employed to determine the effect of vehicle and formulation on skin sensitising potency (Warbrick *et al*, 1999b; Lea *et al*, 1999). Also, it has been demonstrated that the derivation of EC3 values from LLNA responses is stable with time and between laboratories (Dearman *et al*, 1998; Warbrick *et al*, 1999a). Finally, it must be emphasised that the vehicle used may influence EC3 values and that this needs to be appreciated in comparing relative potencies.

The approach taken to derive EC3 values is to estimate mathematically from LLNA dose-response analyses the amount of chemical necessary to elicit a stimulation index (relative to vehicle treated controls) of 3. For this purpose, of the statistical approaches available, it is recommended that EC3 values are derived by linear interpolation between values on LLNA dose response curves that fall either side of the 3-fold stimulation index (Basketter *et al*, 1999d). Using this approach, the EC3 value is determined by linear interpolation of points on the dose-response curve immediately above and below the 3-fold threshold. The equation used for calculation of EC3 is:

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

where:
a = the lowest concentration giving stimulation >3;
b = the actual stimulation index caused by a;
c = the highest concentration failing to produce a stimulation index of 3;
d = the actual stimulation index caused by c.
It should be noted that the vehicle control data (stimulation index = 1) should not be used for co-ordinates c and d.

Taken together, the available data indicate that EC3 values derived from LLNA responses provide a realistic approach to determination of the relative skin sensitising potency of chemicals. Thus, in a recent analysis it was shown that a group of contact allergens could be ranked in terms of sensitising activity on the basis of derived EC3 values. In those investigations it was suggested that, in practice, the most appropriate approach might be to classify the potency of chemicals into groups based upon derived EC3 values expressed in terms of percentage or molar application concentrations (Basketter *et al*, 2000). Using this approach one possible criterion would be to group chemicals into the following potency categories: EC3 <0.1% application concentration, EC3 0.1% - 1.0%, EC3 1.0 - 10% and EC3 >10%. However, this suggestion serves to illustrate just one approach and other strategies may be equally appropriate. Examples illustrating the application of EC3 values for comparing the inherent sensitising potential of chemicals, and for comparing the influence of vehicle matrices are shown below.

3.3 Examples

The two examples illustrated below derive from recent investigations. In the first case the relative skin sensitising potency of two different but related chemicals (A and B) were determined when formulated in the same vehicle. The relative skin sensitisation potencies of A and B were investigated when both materials were formulated in acetone. In both cases dose-response analyses were performed. The results are summarised in Table 8 where the stimulation indices obtained are recorded as a function of the percentage application concentration of the test chemicals. From these analyses EC3 values were derived by linear interpolation. Chemical A had an EC3 value of 0.43%. The EC3 value for chemical B was 0.09%. From these results it was concluded that, when formulated in acetone, chemical B displayed approximately five times greater skin sensitisation potency than did chemical A on the basis of percentage application concentration. Evidence available from a review of occupational allergic contact dermatitis to these chemicals indicates that chemical B has a greater potential for human sensitisation than does chemical A (Hilton *et al*, 1998).

Table 8: Potency assessment using the LLNA: a comparison of chemicals A and B

Chemical A		Chemical B	
Application conc (% in acetone)	**Stimulation indices**	**Application conc (% in acetone)**	**Stimulation indices**
0	1.0	0	1.0
0.093	1.1	0.05	1.3
0.185	2.3	0.125	4.3
0.37	2.9	0.25	7.6
0.93	3.9	0.5	11.6
1.85	4.0	1.25	17.7
		2.5	18.0
EC3 = 0.43%		**EC3 = 0.09%**	

In the second example, the influence of vehicle on the relative skin sensitisation potential of a third, unrelated chemical (C) was determined. Chemical C was applied in either acetone or in propylene glycol and LLNA dose-response analyses conducted. The results are summarised in Table 9 where the stimulation indices obtained are again recorded as a function of the percentage application concentration of each formulation. When dissolved in acetone, chemical C was found to yield an EC3 value of 0.008%. The EC3 value for chemical C in propylene glycol was 0.048%. From these data it was concluded that when formulated in acetone, chemical C displayed approximately six times greater skin sensitisation potency (in terms of percentage application concentration) than when formulated in propylene glycol.

Table 9: Potency assessment using the LLNA: evaluation of chemical C applied in acetone or propylene glycol

Application concentration (%)	Stimulation indices	
	Acetone	**Propylene glycol**
0	1.0	1.0
0.0015	1.2	2.0
0.0075	2.9	0.8
0.015	9.3	2.1
0.0375	17.7	2.3
0.075	23.5	4.7
	EC3 = 0.008	**EC3 = 0.048**

3.4 Advantages and Limitations

Based upon the above considerations, it is concluded that the LLNA provides an appropriate and a realistic approach to determination of the relative skin sensitising potency of chemicals and formulations. Clearly, however, a more detailed examination of the relationship between derived EC3 values and known differences in skin sensitising activity will be required before formal endorsement of the LLNA for potency assessment can be granted. Notwithstanding the need for formal ratification, the view is that the LLNA currently represents the method of choice for deriving estimates of inherent skin sensitising potency for the purposes of risk assessment. The advantages and limitations of the use of the LLNA for potency assessment are summarised below:

Advantages:

- The LLNA is relatively rapid, cost-effective and, compared with guinea pig tests, confers important animal welfare benefits;
- The end-point measured is necessary for, and correlates closely with, the acquisition of skin sensitisation.
- An index of potency (EC3 value) can be derived directly from mathematical interpolation of LLNA dose-response analyses;
- Experience to date reveals that there exists a close association between derived EC3 values and what is known of the relative skin sensitising potential of chemicals among humans.

Limitations:

- The use of EC3 values derived from LLNA dose responses has not yet been validated extensively or endorsed formally.
- The standard LLNA cannot be used for evaluating potency at the elicitation stage.

4. HUMAN SKIN SENSITISATION TESTING

4.1 Background

The testing and risk assessment process for skin sensitisation of finished products and formulations demands consideration of several important factors relevant to both occupational and consumer use exposures (Robinson *et al*, 1989). One factor is the influence of formulation components (matrix effects) on the bioavailability and sensitising activity of potentially allergenic ingredients. In practice, the testing of finished products in animals is seldom conducted except to investigate rare situations in which known skin sensitisers are formulated with unique excipients that could increase sensitisation potential, or to address regulatory requirements for marketing. The testing of finished products is confined to human methods and these are intended to confirm the safety of products once ingredient testing has indicated that the formulation is safe for human exposure. Human skin sensitisation testing is, however, more common for products that are intended to come into contact with skin, rather than for industrial chemicals. The potential exacerbating effects of formulation components on skin penetration and bioavailability of an ingredient, if relevant, are most often addressed in the determination of the allowable exposure limits for the human test.

In this Section, the focus is on the Human Repeat Insult Patch Test (HRIPT). The human maximisation test, which is clearly less appropriate for investigations of potency and thresholds, is not considered.

4.2 Human Skin Sensitisation Testing and Potency Assessment of Chemicals and Formulations

Provided that the risk of inducing skin sensitisation in volunteers is judged to be minimal (based on assessment of pre-clinical sensitisation data), human testing may be conducted. The principles that must be followed prior to the initiation of such studies have been outlined in a recent paper which also puts these principles into the context of European legislation on chemicals and preparations (Roggeband *et al*, 1999).

It is important to emphasise that an HRIPT, or any other type of human skin sensitisation test, is performed to confirm safety under exaggerated conditions of product exposure. The HRIPT is not conducted to identify skin sensitisation hazards. Obviously, however, if a panellist is found to be sensitised, this must be taken into account in the risk assessment process. Neither is the HRIPT designed to obtain information about the potency of a skin sensitiser. It is possible, however, to compare for example, the highest level of known sensitisers not resulting in contact skin sensitisation (Basketter *et al*, 1997b), and these data are valuable in a risk assessment context. Clearly, even potent sensitisers can be used safely, provided that the risk assessment is favourable (Fewings and Menné 1999; Basketter *et al*,1999 a and c).

Methodological aspects of the HRIPT have been described in detail elsewhere (Stotts, 1980). The concentration at which a chemical is evaluated is determined by integrating such key factors as prior sensitisation test results in animals, results from repeated application irritation patch studies in humans, the desire to exaggerate exposure relative to intended use/other anticipated uses (if irritancy considerations permit) and prior experience. It is sometimes preferred that a material be tested at the highest minimally irritant concentration as determined in a human irritation screen. Skin sensitisation reactions in volunteers are characterised usually by erythema coupled with one or more of various dermal sequelae such as oedema, papules, vesicles, bullae and/or pruritis Challenge is performed concurrently at both the original and alternate (naïve) skin sites. A response that occurs and persists at both challenge sites is indicative of skin sensitisation and should be confirmed by an appropriate challenge. The challenge of both the original and naïve sites, the scoring timeline, and the rechallenge procedure maximise the sensitivity and reliability of the test procedure.

The HRIPT also detects pre-existing sensitisation to the test material when there are persistent reactions, characterised as above, early during the induction period. Any positive or questionable response during the induction phase, or only during the challenge phase, needs to be investigated further by a rechallenge patch study. A dose-response study may also be conducted to determine whether a threshold for elicitation can be established. In addition, skin sensitisers in formulations may be identified via rechallenge. However, this is limited to exceptional cases. In the vast majority of HRIPT studies volunteers will not be sensitised.

Procedures for conduct of HRIPTs may need to be modified on occasion to address specific chemical or product formulation characteristics. For example, formulations with volatile components intended for non-occluded use may be too irritant for fully occluded patch testing. An open application or semi-occluded patch may be used instead. In cases where exposure may not provide adequate exposure exaggeration (for example transdermal drugs), smaller scale tests with fewer subjects are appropriate (Robinson *et al*, 1991).

4.3 Examples

In human testing, the hallmarks of skin sensitisation are 1) reactions that are oedematous or papular, 2) the persistence or increase in severity of the reactions over time, and 3) reactions at both the original and naïve (alternate) sites. Sensitisation reactions are most frequently erythematous, papular, and oedematous. Conversely, primary irritation reactions (unless severe), are generally erythematous only. An irritant reaction is usually uniform with a well-defined border, whereas an allergic response (especially if weak), is typically non-uniform, can often have an irregular border, and a strong response may spread beyond the patch site. In cases of true sensitisation, responses should develop at both the original and alternate sites and persist through two delayed scoring timelines at least 24 hours apart. Challenge reactions that subside in severity from the 48 to 96 hour grading period are considered usually to be due to primary irritation, rather

than to skin sensitisation. Responses which are more severe at challenge than during early induction are suggestive of skin sensitisation. Responses that increase or maintain severity from the 48 to 96 hour challenge gradings are indicative of skin sensitisation. Persistent skin responses with papules and/or oedema occurring in the first week of induction suggest pre-existing skin sensitisation. Similar reactions that occur later in induction suggest *de novo* sensitisation.

The importance of rechallenge in skin sensitisation testing has been addressed above. In an HRIPT rechallenge, patches are applied to the original site and a naïve alternate site on the opposite side of the body for 24 hours. Often, however, only a new naïve site is used at rechallenge (e.g. on the back). The sites are graded for skin responses 48 and 72 hours, or 48 and 96 hours, after patch application. The original test material is always evaluated in the rechallenge. Note that ingredients themselves may also be tested in the rechallenge, at a previously determined non-irritant concentration. In conducting a rechallenge, it may be appropriate to include control subjects(s) as well as the treated subjects to establish a norm (or benchmark) for primary irritation to assist with data interpretation. A rechallenge can be performed between 4 and 12 weeks after the initial challenge. This delay allows previous reactions to subside, and is the optimum time for confirmation of the presence or absence of skin sensitisation. As is true for all skin sensitisation studies, rechallenge data must be interpreted with care and within the context of the entire study. The interpretation has to be performed on a case-by-case basis, using as a guide the best understanding of immunology integrated with the knowledge of those who have substantial experience in conducting the test.

Table 10 depicts the threshold dose (per unit area of skin) for five contact allergens of markedly different potencies in both the HRIPT and the LLNA. Using dose per unit area, similar thresholds can be demonstrated for these different chemicals in both species, something that would not be apparent if total percentage concentration was used as the exposure constant (Robinson *et al*, 2000). From Table 10 it is clear that the highest level tested in the HRIPT that resulted in no sensitisation corresponds well with the EC3 value in the LLNA.

Table 10 : Contact allergenicity as determined by two different skin sensitisation methods, LLNA and HRIPT†

Test Allergen	HRIPT* (µg/cm²)	Vehicle	References	LLNA EC3** (µg/cm²)	Vehicle	References
MCI/MI***	~1	water; product formulations	Cardin *et al*, 1986 Basketter *et al*, 1999c	2.5	dimethylformamide	Botham *et al*, 1991b Basketter *et al*, 1999c
p-Phenylenediamine	~10	petrolatum	Marzulli and Maibach, 1974	25	acetone/olive oil (4:1)	Basketter *et al*, 2000
Glutaraldehyde	~50	petrolatum	Marzulli and Maibach, 1974	50	acetone/olive oil (4:1)	Basketter *et al*, 2000
Cinnamic aldehyde	~600	ethanol	Danneman *et al*, 1983	500	acetone/olive oil (4:1)	Basketter *et al*, 2000
Hydroxycitronellal	~3000	ethanol/diethyl-phthalate (3:1)	Ford *et al*, 1988	5000	acetone/olive oil (4:1)	Basketter *et al*, 2000

* Highest level tested in HRIPT that resulted in no skin effects

** Lowest dose yielding a positive response (stimulation index ≥ 3) in LLNA

*** MCI/MI : methylchloroisothiazolinone/methylisothiazolinone

† *Adapted from Robinson et al, 2000*

4.4 Advantages and Limitations

The obvious advantage of human skin sensitisation tests is that data are generated in the relevant species and are immediately applicable to risk assessment. Another clear advantage is that the influence of the vehicle or formulation matrix on skin sensitisation can be investigated readily. Useful threshold information can thus be obtained for different product applications.

However, human skin sensitisation testing is not without limitations. Studies involving volunteers must be conducted using the highest ethical and safety standards. Any study involving humans must be interpreted in the context of inter-individual variability. Consumers cover a broad spectrum of individual characteristics in terms of skin types; there are individual studies demonstrating population differences in skin properties or in responsiveness to chemical insult (Robinson, 1999).

5. APPLICATION OF TEST METHODS IN RISK ASSESSMENT

5.1 Background

The risk assessment process integrates the intrinsic toxicity (hazard) of a chemical with likely conditions and levels of exposure. However, in this monograph attention is focused on the application of sensitisation data to potency assessment which represents just one key element of the risk assessment process. Many chemicals in common use possess, to some degree, a potential to cause skin sensitisation. Consequently, it is important to conduct a thorough and accurate risk assessment when it is anticipated that such chemicals are likely to come into contact with human skin. For example, it is well known that sensitising chemicals, such as certain preservatives, can be formulated into consumer products at levels that are of negligible risk for skin sensitisation provided that the exposures are below the recognised thresholds for induction of sensitisation (Cardin *et al*, 1986; Frosch *et al*, 1995). It is equally well established that these same ingredients can trigger sensitisation when formulated into products inappropriately, for instance at too high a level (Hannuksela, 1986).

A risk assessment for skin sensitisation must consider both the nature and extent of skin exposure, together with the sensitising potency of the chemical (Robinson *et al*, 2000). As discussed in previous sections, it is important in skin sensitisation to express exposure in terms of the amount of chemical per unit area of skin. The use of guinea pig tests and the LLNA in risk assessment has been reviewed in detail previously (Gerberick *et al*, 1993; Basketter *et al*, 1996b; Kimber and Basketter, 1997; Basketter, 1998; Gerberick and Robinson, 2000). In this Section, the use of such tests and of human methods in risk assessment is summarised.

5.2 Guinea Pig Tests

Guinea pig tests have been of some use in addressing questions of sensitisation potency and antigenic cross reactivity of structurally related chemicals, identification of chemical contaminants in raw materials, or comparison of the sensitisation potential of a raw material produced by different chemical processes. Data from guinea pig tests of new chemicals are typically compared with those obtained with benchmark materials, with predictive human test results, and with worker and marketplace experience. Results with an unknown chemical are evaluated in relation to data available for known allergens and non-allergens. In effect, a comparison is made between the data generated on the new chemical and results obtained previously with benchmark chemicals of similar structure and/or product use that have either a safe history of manufacture and consumer use or which were judged to be unsafe (Robinson *et al*, 1989 and 1990). The data must be interpreted with respect to the test concentrations, vehicles and the incidence and intensity of any sensitisation reactions. Where a chemical is likely to have extensive skin contact at a significant concentration, only the most sensitive of test methods can be expected to provide an adequate assessment of sensitisation potential (Robinson *et al*, 1991; Robinson and Cruze, 1996).

Frequently there are differences between the occluded patch test of Buehler and the OET compared with the GPMT, that make the former tests more suitable for use in risk assessment. In the OET and Buehler test, exposure is via the relevant route and there is no use of adjuvant. The particular advantage of the OET is that dose-response

relationships for induction and elicitation are considered. What the guinea pig tests have in common is that the design is such that the induction and challenge concentrations are determined by the irritancy of the test material. As a consequence, potency information is limited, except for chemicals that are structurally related. Moreover, guinea pig data are sometimes difficult to interpret in terms of direct extrapolation for skin sensitisation in humans (Basketter *et al*, 1997b).

5.3 Local Lymph Node Assay

Compared with available guinea pig methods, the LLNA offers some important advantages. The end-point measured is objective and not subject to the interpretative difficulties that may confound visual assessment of challenge-induced inflammatory reactions. Also, exposure is via the relevant route. Importantly the concentrations chosen for analysis in the LLNA are not dictated by the irritant properties of the test material. One aspect of the LLNA which makes it particularly attractive compared with guinea pig methods is that it offers the prospect of a more soundly based assessment of sensitising potency, although there is still the possibility of species differences.

The use of EC3 values for relative potency assessments has been proposed (Kimber and Basketter, 1997). Recently EC3 measurements have been compared with an assessment of the sensitising potency of a number of chemicals, each assigned to one of five classes, based on their human sensitising potency. The EC3 values were found to correlate well with the human categorisation, with the strongest sensitisers having low EC3 values, weaker sensitisers having intermediate EC3 values and non-sensitising chemicals having EC3 values reaching infinity. The EC3 value of a chemical therefore provides an objective and quantitative estimate of potency that is of utility for skin sensitisation risk assessment (Basketter *et al*, 2000; Gerberick and Robinson, 2000).

5.4 Human Skin Sensitisation Testing

Evaluation of relative potency represents an important component of any risk assessment process. However, for derivation of safe exposure levels it is necessary to use such data in the context of information available for known human skin sensitisers and the conditions under which these chemicals will be encountered. In practice, this involves comparison of potency with an index chemical for which dose-response relationships with respect to human skin sensitisation are already understood. Such a next step could utilise data deriving from a HRIPT.

One of the critical factors in the process of risk assessment is the influence of formulation components (matrix effects) on the bioavailability and immunological activity of potentially sensitising ingredients. Several examples of the effect that formulations can have on the sensitising potency of chemicals are to be found in the literature (Robinson and Sneller, 1990; Robinson *et al*, 1991; Calvin, 1992; Heylings *et al*, 1996; Robinson and Cruze, 1996; Seidenari *et al*, 1996; Merk, 1998). In practice it is uncommon to test finished products and formulations in animals. This is usually confined largely to human test methods intended to confirm the safety of products once ingredient testing has indicated that the formulation is likely to be safe for human exposure. Provided that the risk of inducing skin sensitisation in volunteers is judged to be minimal, human skin sensitisation testing may be conducted. Confirmation that humans will not respond

adversely provides supplementary information for safety programmes designed to assess the skin sensitisation risk of new chemicals and products, particularly formulated products.

The HRIPT can provide an exaggeration of intended product use through an extended duration of exposure, testing higher than intended use concentrations, administering concentrations that cause mild skin irritation, and through the use of an occluded patch. The data generated in the HRIPT are combined with results from the LLNA and/or guinea pig assays, and information on likely worker and consumer exposure. In some instances, this information can be compared with the data on relevant benchmark materials to give an improved assessment of risk (Robinson *et al*, 1989; Gerberick *et al*, 1993; Gerberick and Robinson, 2000). Depending on the data generated, exposure estimates, and comparison with benchmarks, a decision may be reached to either reconsider the development strategy or to obtain additional clinical data in carefully controlled use tests under more realistic exposure conditions before proceeding with a new chemical ingredient for product development.

In addition to the HRIPT, there exists a number of other tests involving human volunteers, where skin sensitisation can be evaluated. The extended prospective use test is a method for verifying that a product will not cause allergic contact dermatitis under typical conditions of product use over a significant period of time. As in other clinical studies, a formal risk assessment is conducted, written informed consent is obtained, and expert dermatological monitoring and assessment of any skin reactions are included in the protocol (Robinson *et al*, 1989). The number of test subjects, and the length of a prospective use test, are dependent on the product being evaluated. Prospective use tests can comprise 100 to 500 subjects and extend for three to six months. Diagnostic patch testing at the beginning and at the conclusion of the study should be used to verify lack of patch test reactivity or to identify sub-clinical sensitisation responses (i.e. subjects with sensitisation in the absence of clinical effects).

Taken together, the judicious use of animal test methods (and in particular, EC3 values deriving from the LLNA), together with estimates of likely exposure and relevant benchmark data on human sensitisation, provide a sound basis for the development of accurate risk assessments. Such risk assessments can be fortified further by the conduct, where appropriate, of relevant human studies to confirm safe levels of exposure.

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Both guinea pig tests and the LLNA have been used for the purposes of determining the skin sensitising potency of chemicals. Although attempts have been made to reconfigure the GPMT for the purpose of deriving dose-response relationships, this method is usually unsuitable for assessment of relative potency. Other guinea pig tests that do not require the use of adjuvant and which employ a relevant route of exposure (the occluded patch test and the OET) are more appropriate for considering skin sensitising potency. Suitable also is the LLNA which has been adapted for derivation of comparative criteria, such as EC3 values, that provide an effective and quantitative basis for determination of relative skin sensitising potency. It must be emphasised for all of the approaches identified above that potency is measured relative to other chemical allergens of known skin sensitising activity. The estimation of likely threshold concentrations is dependent upon the availability of suitable benchmark chemicals of known potency with respect to human sensitisation. Finally, human testing (and specifically the HRIPT) can help to confirm the absence of skin sensitising activity of formulations and products under specific conditions of use and exposure. On the basis of these conclusions the following recommendations are made:

- If results are already available from suitable guinea pig tests then judicious interpretation of the data may provide information of value in assessing relative skin sensitising potency. This option should be explored before other analyses are conducted.
- The LLNA is the recommended method for new assessments of relative potency, and for the investigation of the influence of vehicle or formulation on skin sensitising potency.
- Whenever available, human skin sensitisation data should be incorporated into an assessment of relative potency.

Clearly, for the purposes of developing accurate risk assessments it is necessary that all available data from animals and humans be considered in an integrated fashion. Appropriate interpretation of existing data from suitable guinea pig tests can help with the determination of potency as a first step in the risk assessment process. However, for *de novo* investigations the LLNA is the method favoured for providing quantitative estimates of skin sensitising potency that are best suited to the risk assessment process. Finally, human testing is of value for risk assessment, but is performed only for the purposes of confirming product safety.

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No. 8 Methyl Isobutyl Ketone
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No. 10 Isophorone
No. 11 1,2-Dichloro-1,1-Difluoroethane (HFA-132b)
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No. 15 1-Fluoro 1,1-Dichloroethane (HFA-141B)
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No. 8 HAZCHEM; A Mathematical Model for Use in Risk Assessment of Substances
No. 9 Styrene Criteria Document
No. 10 Hydrogen Peroxide OEL Criteria Document (CAS No. 7722-84-1)
No. 11 Ecotoxicology of some Inorganic Borates
No. 12 1,3-Butadiene OEL Criteria Document (Second Edition) (CAS No. 106-99-0)
No. 13 Occupational Exposure Limits for Hydrocarbon Solvents
No. 14 n-Butyl Methacrylate and Isobutyl Methacrylate OEL Criteria Document
No. 15 Examination of a Proposed Skin Notation Strategy
No. 16 GREAT-ER User Manual

Documents

No. Title

No. 32 Environmental Oestrogens: Male Reproduction and Reproductive Development
No. 33 Environmental Oestrogens: A Compendium of Test Methods
No. 34 The Challenge Posed by Endocrine-disrupting Chemicals
No. 35 Exposure Assessment in the Context of the EU Technical Guidance Documents on Risk Assessment of Substances
No. 36 Comments on OECD Draft Detailed Review Paper: Appraisal of Test Methods for Sex-Hormone Disrupting Chemicals
No. 37 EC Classification of Eye Irritancy
No. 38 Wildlife and Endocrine Disrupters: Requirements for Hazard Identification
No. 39 Screening and Testing Methods for Ecotoxicological Effects of Potential Endocrine Disrupters: Response to the EDSTAC Recommendations and a Proposed Alternative Approach
No. 40 Comments on Recommendation from Scientific Committee on Occupational Exposure Limits for 1,3-Butadiene
No. 41 Persistent Organic Pollutants (POPs) Response to UNEP/INC/CEG-I Annex 1