ECETOC

Technical Report No. 78

Skin Sensitisation Testing:

Methodological Considerations

Technical Report No. 78

Skin Sensitisation Testing:

Methodological Considerations

Brussels, December 1999

ECETOC Technical Report No. 78

SKIN SENSITISATION TESTING:
METHODOLOGICAL CONSIDERATIONS

CONTENTS

SUMMARY

In this Technical Report various methodological aspects of skin sensitisation testing have been considered with the objective of addressing the following remit:

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 addressed: (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 reviewed.

A number of conclusions have been drawn and recommendations made. The recommendations are as follows:

- 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 in 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 the opportunity for both animal welfare benefits and improved hazard identification.

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 last 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. In addition, it is relevant also to ask whether and in what ways the methods available can be employed for the purposes of 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.*

These remits have been addressed by the Task Force. The deliberations and recommendations of the Task Force are combined within a Technical Report which addresses specifically the first remit and a Monograph that considers both remits.

INTRODUCTION: SKIN SENSITISATION TESTING:
METHODOLOGICAL CONSIDERATIONS

In this Technical Report 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 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 number 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 a net response 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 only be 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 Benefit

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

1.2 JOINT POSITIVE CONTROLS

1.2.1 Background

The OECD Guideline 406 (1992) and the EC Test Method B6 (1996) require regular (every 6 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 6-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 6 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*, 1999). 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.

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 Benefit

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 1-3 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 1-3 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 $\geq$ "1"). In general, a borderline incidence of positive reactions in the test groups (with respect for instance 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. Clearly, the choice of a vehicle is in practice 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 2 to 3 weeks; in fact, weak irritant reactions may not be repeatable after 1 week. In contrast, moderately strong allergic reactions can be evoked at nearly the same intensity for periods of 2 to 3 months. After 5 to 6 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. The 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 a weak, usually homogenous, erythema. Rechallenge was conducted 1 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 rather 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 1 week after challenge 1

In Table 2, the initial challenge indicated that 3 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*, 1996).

■   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*, 1999).

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 (re-challenge).
- Reduction in the incidence of "false positive" and "false negative" results.

BIBLIOGRAPHY

Basketter DA and Gerberick GF, 1996. An interlaboratory evaluation of the Buehler test for the identification and classification of skin sensitizers. Contact Dermatitis 35, 146-151.

Basketter DA, Gerberick GF, Kimber I and Loveless SE, 1996. The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. Fd Chem Toxicol 34, 985-997.

Basketter DA, Gerberick GF, Kimber I and Willis C, 1999. Toxicology of contact dermatitis. Allergy, irritancy and urticaria. Wiley and Sons, Chichester.

Basketter DA, Scholes EW, Kimber I, Botham PA, Hilton J, Miller K, Robbins MC, Harrison, PTC and Waite SJ, 1991. Interlaboratory evaluation of the local lymph node assay with 25 chemicals and comparison with guinea pig test data. Toxicol Methods 1, 30-43.

Basketter DA, Scholes EW, Wahlkvist H and Montelius J, 1995. An evaluation of the suitability of benzocaine as a positive control skin sensitizer. Contact Dermatitis 33, 28-32.

Basketter DA, Selbie E, Scholes EW, Lees D, Kimber I and Botham PA, 1993. Results with OECD recommended positive control sensitizers in the maximization, Buehler and local lymph node assays. Fd Chem Toxicol 31, 63-67.

Bruynzeel DP, Blomberg-van der Flier M, van Ketel WG van and Scheper RJ, 1983. Depression or enhancement of skin reactivity by inflammatory processes in the guinea pig. Int Archs Allergy Appl Immun 72, 67-70.

Buehler EV, 1965. Delayed contact hypersensitivity in the guinea pig. Arch Dermatol 91, 171-177.

Buehler EV, 1996. Nonspecific hypersensitivity: false-positive responses with the use of Freund's complete adjuvant. Contact Dermatitis 34, 111-114.

ECETOC, 1990. Skin sensitisation testing. Monograph No.14, Brussels.

EC Test Method B6, 1996. Commission Directive 96/54/EC of 30 July 1996, adapting to technical progress for the twenty-second time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Methods for the determination of toxicity, B6. Acute toxicity skin sensitisation. OJ No. L248, 206-212.

Fisher A, 1995. Contact Dermatitis 4th ed. Rietschel RL and Fowler JF (eds), Williams and Wilkins, Baltimore.

Frankild S, Basketter DA and Andersen KE, 1996. The value and limitations of rechallenge in the guinea pig maximization test. Contact Dermatitis 35, 135-140.

Gad SC, Dunn BJ, Dobbs DW, Reilly C and Walsh RD, 1986. Development and validation of an alternative dermal sensitisation test: the mouse ear swelling test (MEST). Toxicol Appl Pharmacol 84, 93-114.

Gerberick GF, Ryan CA, Kimber I, Dearman RJ, Lea LJ, and Basketter DA, 1999. Local lymph node assay: validation assessment for regulatory purposes. Am J Contact Dermatitis (In press).

Hofmann T, Diehl K-H, Leist K-H and Weigand W, 1987. The feasibility of sensitization studes using fewer test animals. Arch Toxicol 60, 470-471.

ICCVAM, 1999. The murine local lymph node assay: a test method for assessing the allergic contact dermatitis potential of chemicals/compounds. NIH No. 99-4494.

Jokipii AMM and Jokipii L, 1973. Dose response relationships and interference of simultaneous skin tests in delayed hypersensitivity. J Clin Path 26, 687-690.

Kimber I, 1996. The local lymph node assay. In: Dermatotoxicology 5th ed. Marzulli FN and Maibach HI (eds). Taylor and Francis, Washington DC, 469-475.

Kimber I and Basketter DA, 1992. The murine local lymph node assay: a commentary on collaborative studies and new directions. Fd Chem Toxicol 30, 165-169.

Kimber I and Dearman RJ, 1991. Investigation of lymph node cell proliferation as a possible immunological correlate of contact sensitizing potential. Fd Chem Toxicol 29, 125-129.

Kimber I, and Dearman RJ, 1996. Contact hypersensitivity: immunological mechanisms. In: Toxicology of contact hypersensitivity. Kimber I and Maurer T (eds). Taylor and Francis, London, 4-25.

Kimber I, Dearman RJ, Scholes EW and Basketter DA, 1994. The local lymph node assay: developments and applications. Toxicol 93, 13-31.

Kimber I, Hilton J, Botham PA, Basketter, DA, Scholes EW, Miller K, Robbins MC, Harrison PTC and Gray TJB, 1991. The murine local lymph node assay. Results of an inter-laboratory trial. Toxicol Lett 55, 203-213.

Kimber I, Hilton J, Dearman RJ, Gerberick CF, Ryan CA, Basketter DA, Lea L, House RV, Ladics GS, Loveless SE and Hastings KL, 1998. Assessment of the skin sensitisation potential of topical medicaments using the local lymph node assay: an inter-laboratory exercise. J Toxicol Environ Hlth 53, 563-579.

Kimber I, Hilton J, Dearman RJ, Gerberick GF, Ryan CA, Basketter DA, Scholes EW, Ladics GS, Loveless SE, House RV and Guy A, 1995. An international evaluation of the murine local lymph node assay and comparison of modified procedures. Toxicol 103, 63-73.

Kimber I, Hilton J and Weisenberger C, l989. The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. Contact Dermatitis 21, 2l5-220.

Kimber I, Mitchell JA and Griffin AC, 1986. Development of a murine local lymph node assay for the determination of sensitizing potential. Fd Chem Toxicol 24, 585-586.

Kligman AM and Basketter DA, 1995. A critical commentary and updating of the guinea pig maximization test. Contact Dermatitis 32, 129-134.

Kligman AM and Epstein W, 1975. Updating the maximization test for identifying contact allergens. Contact Dermatitis 1, 231-239.

Loveless SE, Ladics GS, Gerberick GF et al, 1996. Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. Toxicol 108, 141-152.

McGuire RL and Fox RA, 1979. Suppression of delayed hypersensitivity by the depletion of circulating monocytes. Immunol 38, 157.

Magnusson B and Kligman AM, 1969. The identification of contact allergens by animal assay. The guinea pig maximization test. J Invest Dermatol 52, 268.

Magnusson B and Kligman AM, 1970. Allergic contact dermatitis in the guinea pig. Charles C Thomas, Springfield IL.

Maurer T, 1996. Guinea pig predictive tests. In: Toxicology of Contact Hypersensitivity. Kimber I and Maurer T (eds). Taylor and Francis, London,107-126

Middleton E, Ellis EF, Yunginger JW, Reed CE, Adkinson NF and Busse WW, 1998. In: Allergy and Principles. Volumes I and II, 5th ed, Mosby-Year Book Inc.

OECD Guidelines for Testing of Chemicals, 1992. Guideline No. 406. Skin Sensitisation.

Prater E, Göring HD and Schubert H, 1978. Sodium lauryl sulfate – a contact allergen. Contact Dermatitis 4, 242.

Prinsen MK, Romijn T and Snoeij NJ, 1997. Skin sensitization testing: the relevance of rechallenge and pretreatment with sodium lauryl sulfate in the guinea pig maximization test. Fd Chem Toxicol 35, 923-926.

Prinsen MK, Romijn T and Snoeij NJ, 1999. Skin sensitization testing: the relevance of rechallenge and pretreatment with sodium lauryl sulfate in the guinea pig maximization test. Letters to the Editor. Fd Chem Toxicol 37, 267-268.

Robinson MK, Nusair TL, Fletcher ER and Ritz HL, 1990. A review of the Buehler guinea pig skin sensitization test and its use in a risk assessment process for human skin sensitization. Toxicol 61, 91-107.

Robinson MK, Stotts J, Danneman PJ, Nusair TL and Bay PHS, 1989. A risk assessment process for allergic contact sensitization. Fd Chem Toxicol 7, 479-489.

Sams WM and Smith JG, 1957. Contact dermatitis due to hydrocortisone ointment. J Am Med Assoc 164, 1212.

Scholes EW, Basketter DA, Sarll AE et al, 1992. The local lymph node assay: Results of a final inter-laboratory validation under field conditions. J Appl Toxicol 12, 217-222.

Shillaker RO, Bell GM, Hodgson JT and Padgham MDJ, 1989. Guinea pig maximisation test for skin sensitisation: the use of fewer test animals. Arch Toxicol 63, 283-288.

Stotts J, 1980. Planning, conduct, and interpretation. Human predictive sensitisation patch tests. In: Current concepts in cutaneous toxicity. Academic Press, 41-53.

Stropp G, 1998a. Guinea pig maximization test (GPMT): Excited skin syndrome – 2 examples. 4th Congress of the European Society of Contact Dermatitis. Helsinki.

Stropp G, 1998b. Validation of the Magnusson-Kligman maximization test method used by the Fachbereich Toxikologie, Bayer AG performed in guinea pigs of the strain Hsd Poc: DH with 2-mercaptobenzthiazole. Study No. T 1062427 (unpublished study report).

Stropp G, 1998c. Validation of the Magnusson-Kligman maximization test method used by the Fachbereich Toxikologie, Bayer AG performed in guinea pigs of the strain Hsd Poc: DH with 2-mercaptobenzthiazole. Study No. T 6062233 (unpublished study report).

Stropp G, Berthold K, Bury D, Heusener A, Kühlem C and Pittermann W, 1999. Skin sensitization testing: the relevance of rechallenge and pretreatment with sodium lauryl sulfate in the guinea pig maximization test. Letters to the Editor. Fd Chem Toxicol 37, 266-267.

Uehara M and Ofuji S, 1977. Supressed cell-mediated immunity associated with eczematous inflammation. Acta Derm (Stockholm) 57, 137-139.

Wilhelm KP, Surber C and Maibach HI, 1991. Effect of sodium lauryl sulfate-induced skin irritation on in vivo percutaneous penetration of four drugs. J Invest Dermatol 97, 927.

MEMBERS OF THE TASK FORCE

I. Kimber (Chairman)   AstraZeneca
                                                                    UK-Macclesfield

D. Basketter   Unilever
                                                                    UK-Port Sunlight

K. Berthold   Asta Medica
                                                                    D-Halle-Künsebeck

J-.L. Garrigue   L'Oréal
                                                                    F-Aulnay-Sous-Bois

L. Lea   Unilever
                                                                    UK-Bedford

C. Newsome   Union Carbide
                                                                    B-Anvers

R. Roggeband   Procter & Gamble
                                                                    B-Strombeek-Bever

W. Steiling   Henkel
                                                                    D-Düsseldorf

G. Stropp   Bayer
                                                                    D-Wuppertal

S. Waterman *   Exxon
                                                                    USA-East Millstone

C. Wiemann   BASF
                                                                    D-Ludwigshafen

M. Butler   ECETOC
                                                                    B-Brussels

* Corresponding member

MEMBERS OF THE SCIENTIFIC COMMITTEE

(Peer Review Committee)