MONOGRAPH No. 14

SKIN SENSITISATION TESTING

CONTENTS

ECETOC Monograph No. 14

FOREWORD

Up to now the European Chemical Industry Ecology and Toxicology Centre (ECETOC) has published thirteen Monographs in which it has attempted to clarify and express its views on some of the more important problems in toxicology. This Monograph is a further addition to the series.

Skin sensitisation (also known as allergic contact dermatitis) is an important occupational and public health concern. It is therefore important to determine the potential of any new substance to cause sensitisation. A number of methods, all using the guinea pig as the experimental model, have been developed over the last 30 years, seven of which have been included in regulatory guidelines. The aim of the present Monograph assesses the current status and examines how the assessment of skin sensitisation potential could be changed to allow improved risk assessment, both by modifying current test methods and by introducing well validated, alternative test methods. The role of predictive patch testing using human volunteers was also considered.

It is with pleasure that I present this Monograph to all those who are concerned with both human and animal welfare.

R.R. Knowland

Chairman of ECETOC Board

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Skin sensitisation (also known as allergic contact dermatitis) is an important occupational and public health concern. It has been estimated that in the USA, 46% of occupational diseases are skin conditions and about 20% of these are of allergic origin. It is therefore important to determine the potential of any new substance to cause sensitisation. This monograph reviews current methods and alternative approaches for assessing skin sensitisation potential. The following conclusions have been reached:

With the exception of the Draize Test, the guinea pig test methods currently accepted by regulatory authorities worldwide are well able to predict the potential of a material to cause skin sensitisation. Nevertheless,

a) some methods are more sensitive than others (e.g. adjuvant tests are generally more sensitive than non-adjuvant tests),

b) methods cannot be sufficiently standardised to give full reproducibility of results between laboratories,

c) most methods are based on subjective visual grading of skin reactions; difficulties thus arise when testing coloured or irritant materials.

Laboratories must be able to show the sensitivity of the method(s) they use by demonstrating that positive reactions occur with mild/moderate contact allergens rather than the strong/extreme sensitisers currently recommended in certain guidelines, specifically in the EEC Test Method.

The sensitivity of the adjuvant tests is such that it is possible to halve the minimum number of animals required by present regulatory guidelines without compromising their capacity to detect weak/mild sensitisers. A similar review has not yet been made for non-adjuvant tests.

Alternative test methods, including some recently developed mouse models offer several advantages, including the possibility of more objective end points. These tests have not been extensively validated and this precludes their use at present for regulatory purposes other than to confirm the sensitisation potential of a material. Two new test methods employing mice, the Mouse Ear Swelling Test (MEST) and the Local Lymph Node Assay (LLNA), appear promising. They should undergo rigorous inter-laboratory testing to determine their sensitivity and specificity.

In vitro methods do not represent a viable alternative in the foreseeable future. An approach using quantitative structure activity relationships (QSAR) is the most likely route to a non-animal model, but this will require considerable research, development and validation.

Human sensitisation tests have generally not been used for the classification of substances as non-sensitisers. This is because of an absence of internationally agreed test protocols, the difficulties in including positive controls and because the methods for establishing the sensitivity of human tests are less developed than for animal tests. Nevertheless for products for which direct human contact is intended, predictive tests in human volunteers can be considered.

The sensitisation potential of a substance, as determined using a test model, has relevance to man only when interpreted in relation to likely human exposure.

The EEC directive for the classification, packaging and labelling of dangerous substances provides a reasonable approach to the evaluation of skin sensitisers. In contrast, one of the options to meet the requirements of the EEC Dangerous Preparations Directive that preparations containing $\geq$ 1% of a sensitiser should be labelled, fails to take into account the potency of different sensitisers and the fact that incorporation of weak sensitisers into formulations at a concentration $\geq$ 1% may not constitute a sensitising hazard.

A. INTRODUCTION

Jadassohn (1895) was the first investigator to use the patch test to assess contact allergy in man. Whilst other clinicians followed his lead, it was almost half a century before predictive studies in animals and man were described (Draize et al., 1944; Schwartz and Peck, 1944; Shelanski and Shelanski, 1953). The development of predictive tests was based on studies of cellular immunology, which were conducted largely in the guinea pig (Landsteiner and Jacobs, 1935, 1936; Landsteiner and Chase, 1937, 1941, 1942). Draize modified his original protocols during the next two decades (Draize, 1959) and others proceeded to develop predictive assays in the guinea pig (Buehler, 1965; Magnusson and Kligman, 1969) and in man (Kligman, 1966; Marzulli and Maibach, 1974; Stotts, 1980). This work is discussed in this monograph.

A number of methods, all using the guinea pig as the experimental model, have been developed over the last 30 years. Seven of them have been included in regulatory guidelines; Magnusson and Kligman Maximisation Test, Draize Test, Freund's Complete Adjuvant Test, Maurer Optimisation Test, Buehler Test, Open Epicutaneous Test and Split Adjuvant Test. The OECD Guideline No. 406 (OECD, 1981), Test Method B6 of the European Community (EEC, 1984) and the Japanese Agricultural Chemicals Laws and Regulations (Japan/MAFF, 1985) refer to methods for detecting the potential of a chemical substance to cause skin sensitisation. The EEC accepts all seven methods at present and recommends that *"practical considerations"* should determine the selection of the most relevant. The Magnusson and Kligman test is preferred as *"its sensitivity and ability to detect potential human skin sensitisers are considered important in a classification for toxicity relevant to public health"*. Subdivision F of the pesticide assessment guidelines (US-EPA/FIFRA, 1984) also lists seven methods but the Draize Test is omitted and the Footpad Technique is included.

Although these tests are used for labelling purposes (e.g. in the EEC under Annex VI of the Dangerous Substances Directive), there is no international agreement on the conduct of the tests or on the interpretation of the test

results in terms of human hazard and risk. Nor is there an agreed view, based on data from a wide range of chemicals, on the relative sensitivities of the tests, except that adjuvant tests are in general more sensitive than non-adjuvant procedures.

Despite the present uncertainties, practical experience has shown that these guinea pig models can be used successfully in protecting the public from allergic contact dermatitis.

Schlede et al. (1987) have suggested that the number of test procedures accepted by authorities should be reduced to four (two adjuvant and two non-adjuvant tests), that the number of animals used in each test could be reduced and that a two stage testing scheme could be considered (an adjuvant test to estimate the sensitising potential of a test substance followed, if necessary, by a non-adjuvant test *"for classification purposes"*). In parallel with these and other initiatives, several alternative test protocols for assessing skin sensitisation potential have been published, many using the mouse rather than the guinea pig. Some of these tests offer the possibility of more objective and quantitative end points with fewer problems of interpretation caused by the irritancy or colour of the test substance. The reproducibility of these tests in different laboratories has yet to be established.

A major objective of this ECETOC Task Force was to achieve a common view on how the assessment of skin sensitisation potential could be changed to allow improved risk assessment, both by modifying current test methods and by introducing well validated, alternative test methods. The role of predictive patch testing using human volunteers was also considered.

The Task Force was given the following Terms of Reference:

1. review the use of the seven guinea pig tests currently listed in OECD guidelines, commenting upon factors which govern the choice of test;

2. discuss the role of these tests in human risk assessment and propose modifications to the protocols which would allow improved risk assessment;

3. review the range of other tests which are available for assessing skin sensitisation potential, and determine for each its stage of development and validation, and

4. recommend a strategy for accelerating the development and validation of alternative tests, for example, by means of inter-laboratory trials.

Appendix 1 lists and defines the most important technical terms used in this monograph.

B. BACKGROUND

Skin sensitisation, also known as contact sensitivity or allergic contact dermatitis, is a T-lymphocyte mediated delayed hypersensitivity reaction. Details of the mechanism of skin sensitisation can be found in the reviews of Breathnach (1986), Abel and Wood (1986) and Thestrup-Pedersen et al. (1989).

The immunological events in skin sensitisation can be separated into two main phases; the development of sensitisation and the elicitation of clinical effects (e.g. erythema and oedema) following subsequent exposure to the same chemical.

Within the epidermis of mammals there exists a network of dendritic cells, the majority of which, in both the guinea pig and man, are Langerhans cells. These cells interact with the chemical in the skin to form an antigen and actively transport it via the afferent lymphatics to the lymph node draining the site of application.

Within the paracortex of the lymph node, the foreign chemical becomes associated with transmembrane glycoproteins (known as class II histocompatibility determinants) of dendritic cells. The importance of these molecules is that T-lymphocytes can recognise foreign material only when it is associated with class II determinants. From the large number of T-lymphocytes which are constantly migrating through the node, only those few T-cells which have complementary receptors for the antigen undergo activation and rapid proliferation. The result is a selective expansion of antigen reactive clones of T-cells. Although a proportion of these cells differentiate into effector cells whose physiological role is to eliminate antigen, many become quiescent. These so called memory cells are distributed throughout the body by normal routes of lymphocyte traffic but they retain the receptor necessary for interaction with the particular antigen. It is at this point that the individual is sensitised. If re-exposure to the chemical occurs, the memory T-cell will recognise the antigen formed at the site of application and will become activated. The resulting effector lymphocytes manufacture and export a family of protein and glycoprotein molecules, identified collectively as lymphokines, which influence the functional

activity of other cells including those of the immune system. Of particular importance is the ability of such lymphocytes to recruit to the site of exposure other lymphocytes and macrophages and to induce the latter population to become activated and non-selectively aggressive. The mononuclear cell infiltration and local tissue destruction initiated by activated macrophages results in a local inflammatory reaction which is recognised clinically as allergic contact dermatitis.

The cellular and molecular interactions which occur during the induction and elicitation of contact sensitivity are considerably more complex than this synopsis might suggest and are currently the focus of research programmes in both academic and industrial laboratories.

C. ANIMAL TESTS CURRENTLY IN USE

1. *INTRODUCTION*

In the following sections, the guinea pig sensitisation test procedures which are included in the OECD (1981), EEC (1984), US-EPA/TSCA (1985), US-EPA/FIFRA (1984) and Japan/MAFF (1985) methods and guidelines are reviewed. Additional methods have been published in the last 20 years. They are discussed by Maurer (1983), Andersen and Maibach (1985 a, b) and Andersen (1987) and are listed in Appendix 2; although frequently used in investigative or non-regulatory studies, they are generally not used for registration and/or notification of new compounds.

2. *OFFICIAL TEST METHODS AND GUIDELINES*

2.1. General Principles of the Test Methods

During the initial exposure (induction phase) the guinea pigs are treated with intradermal and/or epidermal applications of the test compound. A single re-exposure (challenge phase) to the same test compound is normally performed after a rest period of 10 to 14 days. Sensitisation is determined by examining the skin reactions to a challenge exposure with the maximum non-irritant concentration (epidermal challenge) or by comparing skin reactions following induction and challenge in individual animals (as with the intradermal challenge in the Optimisation Test).

2.2. OECD - Test Guideline (TG) No. 406, Skin Sensitisation (1981)

This guideline contains seven methods, the specific features of which are described in Appendix 3. The main aspects of these methods are listed below.

Test	Mode of induction application	Number of applications	Mode of challenge application
Draize Test	intradermal	x10	intradermal
Freund's Complete Adjuvant Test	intradermal in adjuvant	x5	epidermal open
Optimisation Test (OPT)	1) intradermal 2) intradermal in adjuvant	x4 x6	1) intradermal 2) epidermal (occluded)
Maximisation Test (Magnusson and Kligman) [(M&K)]	1) (a) intradermal (b) intradermal in adjuvant (c) adjuvant 2) epidermal (occluded)	x2 x2 x2 x1	epidermal (occluded)
Split Adjuvant Test (SAT)	1) epidermal (occluded) 2) adjuvant 3) epidermal (occluded)	x2 x1 x2	epidermal (occluded)
Buehler Test	epidermal (occluded)	x3	epidermal (occluded)
Open Epidermal Test (OET)	epidermal (open)	x21	epidermal (open)

Note : the "Footpad Technique for Evaluating Sensitisation Potential in Guinea Pigs is mentioned only in Annex of the OECD Test Guideline.

2.3. EEC Test Method B 6. - Acute Toxicity, Skin Sensitisation (1984)

This test method is part of Annex V (EEC, 1984) of the EEC Dangerous Substances Directive (1979) and is also referred to in the Dangerous Preparations Directive (1988).

The Maximisation Test referred to in the OECD (1981) Test Guideline (TG) is recommended and all general remarks on animals, dose levels and procedure are related to the maximisation protocol of Magnusson and Kligman (1969, 1970). One deviation from the original protocol is that no sodium lauryl sulphate (SLS) pretreatment is requested in the second week of induction for compounds which have no primary irritant effect. The other six OECD procedures are mentioned at the end of the Test Method. These are accepted by the EEC, but *"they must be governed by criteria which ensure that it is valid. Among these criteria is an expected response to standard allergens such as 2,4-dinitrochlorobenzene (DNCB) or p-phenylenediamine (PPD) or another potent sensitiser appropriate to the class of substance being tested" (loc.cit).*

Further remarks are made regarding the choice of any of these six additional methods :

- the selection of a certain method may depend on the physical properties of the test substance,

- sensitisation is potentiated by the injection of Freund's Complete Adjuvant (FCA) and that, therefore, the tests should be divided into groups of adjuvant and non-adjuvant tests and,

- in certain cases, e.g. simulation of human exposure, topical application of the compound is appropriate.

2.4. USA Guidelines

In the US-EPA/TSCA (1985) and US-EPA/FIFRA (1984) guidelines, the list of tests differs from the OECD TG (1981) in that the Draize Test is not included and that the Footpad Test mentioned in the Annex of the OECD guideline is included without further details. The methods are not described but reference is made to the appropriate original publications. No single test is especially recommended.

2.5. Japan MAFF (Agricultural Chemicals Laws and Regulations, 1985)

The section on "dermal sensitisation study" is short. It includes the same list of seven sensitisation methods in tabulated form as in the OECD guidelines. Unlike the EEC test method, no special recommendation is made for a single test.

An important difference from the other guidelines is that mention is made that *"the end-use product should be used as the test substance"*.

D. EXISTING METHODS USING HUMAN VOLUNTEERS

1. INTRODUCTION

Sensitisation test methods using human volunteers reviewed here focus on methods which are regularly used for the development of risk assessments. None of the methods are formally listed in regulatory guidelines in Europe, North America or Japan. Nevertheless, data generated by these and similar methods have been included in reviews of the sensitisation potential of chemicals or preparations by regulatory agencies in many parts of the world. It should be noted that, in contrast to animal models, human studies have not been used as frequently and have not reached a similar degree of international recognition.

2. EXISTING METHODS

2.1. Patch Tests (Stotts, 1985)

All methods in use are based on the same principals as the animal models, i.e. an induction phase to expose the subjects to the test material, a rest period to allow full development of the immune response, and a challenge phase to determine if sensitisation has been induced. Depending on the design of the induction phase, the methods can be divided into three basic groups:

I. Single induction exposure,
II. Repeated induction exposure with rest periods
    a. after each exposure
    b. weekends only
    c. with added insult,
III. Repeated continuous induction exposure.

2.1.1. Single induction exposure. This is exemplified by the original method published by Schwartz and Peck (1944). The skin is exposed to the substance under an occlusive patch for one to four days. After a rest period of 10 - 14 days a challenge patch is applied to a fresh site for 24 - 48 hours. Skin reactions are observed and graded for three days after patch removal. This method is now rarely used.

2.1.2 Repeated induction exposure with rest periods. Test procedures with multiple applications, interrupted by a rest period after each patch, were published by Shelanski and Shelanski (1953), Voss (1958), Draize (1959), Griffith (1969) and Stotts (1980). These methods are often referred to as Human Repeat Insult Patch Tests (HRIPTs). Common to all is a series of 9 - 15 induction exposures, each of 24 hours and followed by a 24 hour period (48 hours over the weekend) during which no patch is worn. After a rest period of (normally) two weeks, the challenge exposure of 24 hours is followed by the observation of skin reactions for up to 72 hours. Occlusive patches are used throughout. The methods differ in the details of the induction and challenge procedures as well as in the method of grading observed reactions. Use of two challenge patches as suggested by Griffith (1969) and Stotts (1980) allows the inclusion of a different lymph node system. Another special feature is the use of a "pilot group" so that adjustments can be made to the test protocol where excessive irritation or allergenicity appears likely. A re-challenge is also recommended after about two months in subjects with questionable reactions.

Pre-treatment of the test site with SLS to increase the sensitivity of the methods was proposed by Kligman (1966) as a "Maximisation Procedure". This method was later updated by Kligman and Epstein (1975) to include an irritation screen (SLS treatment is used only for non-irritant materials) and a pre-test challenge to exclude hyper-irritable subjects or subjects with pre-existing allergies. This is followed by five 48 hour induction patches on the forearm with one or two 24 hour pre-treatments with SLS if the material is not sufficiently irritant. For challenge, a new site is pre-treated with

SLS for one hour followed by a 48 hour application of the test substance. An SLS control is run concurrently. In modifications of this method, stripping of the stratum corneum with adhesive tape or abrasion is used instead of the SLS treatment.

2.1.3. Repeated continuous induction exposure. Methods published by Maibach et al. (1968) and by Jordan (1975) involve exposure to the test material during a three week induction period using ten applications of 48 hours (72 hours over the weekend). Skin reactions are graded between removal of the old and application of the new patch. Challenge consists of exposure to the substance under a patch for 48 or 72 hours, with grading of the response immediately after removal (Maibach et al., 1968), or under two successive 48 hour patches at a new skin site with grading of response up to 72 hours afterwards (Jordan, 1975).

2.2. Open Application / Controlled Long Term Human Use Test

For some materials, such as highly irritant and volatile products, the patch tests described above may not be suitable and could give rise to problems of interpretation of the results. Alternative induction treatments are used for such substances. These usually consist of a preliminary challenge with a low concentration of test material to exclude subjects with pre-existing allergies, followed by daily application of the test substance to relatively large skin areas without occlusive coverage for several weeks. After a rest period the challenge is repeated (Fisher, 1986). Another method for obtaining an indication of a material's sensitisation potential is to conduct an extended controlled product use test (3 to 9 months in duration) with diagnostic patch testing prior to and after the exposure period. This type of test is often included in the follow-up of positive reactions in HRIPTs, both to confirm the nature of the response (sensitisation or irritation) and to assess the relevance of positive HRIPT reactions (Weaver et al., 1985; Müller, 1989; Robinson et al., 1989).

E. COMMENTS ON PRESENT METHODS

1. EXPERIMENTAL METHODS

1.1. General Comments

An earlier survey conducted by ECETOC (1980) on the sensitisation tests used by its members showed that the Maximisation Test was preferred because of its high sensitivity.

A more recent survey performed by ECETOC in several European countries and the USA showed the general trends in sensitisation testing. In Europe, most studies are performed according to the Maximisation Test protocol. The majority of other studies use the Buehler method, whilst the OPT, FCAT and OET are performed much less frequently. Tests which are not in official guidelines are used in some countries for pre-screening purposes or investigative studies but they are not used for registration. In the USA, the Buehler Test is used more frequently than the Maximisation Test.

It is recommended that for the registration or notification of new substances, first consideration should be given to the use of a Maximisation Test or a Buehler Test. It is recognised, however, that there may be circumstances where other test protocols (e.g. OPT, OET) provide the necessary information on sensitisation potential. Furthermore, it is considered that well validated new test procedures could be used for this purpose (cf. Chapter G).

The aim of all sensitisation tests is to determine whether a substance can sensitise and to obtain an indication of its potency. As with other toxicological models the result must be interpreted in the light of a laboratory's experience with appropriate reference compounds. Thus, although the test methods can give rise to a range of results in different laboratories with a single test substance, this need not detract from the value of any particular method. The Task Force considers that it is necessary to define more precisely the requirements

for validation of a method. Experience has shown that the current requirement of the EEC for positive results using potent sensitisers such as DNCB as reference compounds does not allow the relative sensitivity of different test methods to be assessed adequately.

ECETOC recommends that to validate a procedure it is necessary to demonstrate an ability to detect mild/moderate human sensitisers such as benzocaine, neomycin sulphate and hydroxycitronellal (cf. Chapter G).

Positive results from a technique which is less sensitive will need to be interpreted more rigorously. This is reflected in the EEC requirements for classification and labelling (cf. Chapter F).

In the EEC method and OECD guideline the descriptions of the tests are simplified in tabular form and in some cases important features are not included.

ECETOC recommends that, for every method, a list of literature references should be included in guidelines and it should be emphasised that the original literature should also be consulted before performing sensitisation tests.

1.1.1. Selection of induction doses. Magnusson and Kligman (1970) have shown that the results of sensitisation tests are dependent on the dose of chemical administered. Using a number of sensitising chemicals, they employed concentrations of 0.01, 0.1 and 1.0% for epidermal applications and 0.001, 0.01 and 0.1% for intradermal injections. Significant differences were observed in the incidence of positive responses, especially between the lowest and the mid dose. They concluded: *"It may be presumed that every potent allergen has its own characteristic dose response curve with all animals becoming sensitised at the highest dose" (loc.cit).*

Some publications have demonstrated that increasing the induction dose can increase the sensitisation rate but that further dose increases

could result in diminished responses (Roberts and Williams, 1982; Andersen, 1987; Roberts, 1987).

1.1.2. Choice of vehicle. Andersen and Maibach (1985-b) stated : *"In guinea pig allergy tests there is no vehicle that is optimal for all substances"*. The rate of skin penetration of a compound has a major influence on its capacity to induce or elicit sensitisation. Consequently the choice of vehicle may affect the magnitude of the sensitisation response. Similarly, the presence of substances which enhance penetration (e.g. surfactants present in formulations) will influence the sensitisation response.

1.1.3. Evaluation of skin reactions. In all test protocols accepted by regulatory authorities, the evaluation of the epidermal challenge reaction is made by subjective visual scoring of the intensity of erythema and the occurrence of palpable oedema. The exceptions are the assessment of reactions after intradermal injection challenges which are measured in diameter and skinfold thickness increases.

Various methods have been tried to make evaluation of epidermal challenge reactions objective. For example, Andersen and Staberg (1985) utilised measurements of guinea pig skin thickness and laser doppler measurement of blood flow. At present no method using objective parameters has been fully validated. Promising results were, however, obtained by Bratanov et al. (1981) in a mouse method using ear thickness measurements and with histomorphometric evaluations to detect the occurrence of a sensitisation reaction.

1.1.4. Use of Freund's Complete Adjuvant (FCA). FCA stimulates non-specifically the immune system of animals, enhancing the ability of guinea pigs to react to sensitising materials. Therefore test methods using FCA are in general more sensitive than non-adjuvant methods, as recently highlighted by Schlede et al. (1987). Different thresholds for labelling of products have been adopted by the EEC for adjuvant or non-adjuvant methods (cf. Chapter F).

The use of an adjuvant has some disadvantages. FCA itself induces small necrotic reactions. In combination with a strong allergen the necrotic area of different FCA injections may merge to give one large necrotic area. That these undesirable local effects may inhibit sensitisation cannot be excluded.

Magnusson (1980) reported that FCA may lower the irritation threshold of compounds. The use of adjuvant treated control animals is therefore an important step in avoiding a false positive classification of a compound.

It is concluded that adjuvant tests are generally more sensitive than non-adjuvant tests; consequently their continued use is justified.

1.2. Comments on Individual Tests

1.2.1. Draize Test (1944, 1959). Draize attempted to standardise predictive irritation and sensitisation tests. His technique was designed to "screen out" potent sensitisers; negative results have been obtained with known human sensitisers such as benzocaine, mercaptobenzothiazole, neomycin, nickel sulphate and PPD (Buehler, 1965; Magnusson and Kligman, 1969; Prince and Prince, 1977). Another disadvantage of this test is that many features of the protocol are not defined precisely.

1.2.2. Freund's Complete Adjuvant Test. The intradermal injection of the test compound in an adjuvant suspension makes this method more sensitive than the Draize Test. The original method (cf. Appendix 3) was modified by Klecak (1985) to include only three induction treatments and a maximal induction dose of 5 %; although used extensively with fragrances (Klecak, 1985), there are few data on its use with industrial chemicals.

1.2.3. Optimisation Test. This method includes an objective assessment of reactions after intradermal challenge as well as an epidermal challenge. It is a sensitive adjuvant technique designed for active

ingredients but takes longer to perform and is technically more demanding than other methods.

1.2.4. Maximisation Test. This is the most widely used test in Europe and is accepted to be very sensitive; a large number of results have been reported by Wahlberg and Boman (1985). One disadvantage is the occurrence of severe local effects caused by the combination of adjuvant and a high intradermal induction concentration of the test material, followed by an epidermal occlusive application over the injection site.

It should also be noted that the original classification system does not allow any material to be classified as a non-sensitiser (cf Appendix 3; scale 0 corresponds to a weak sensitiser).

1.2.5. Split Adjuvant Test. This technique was designed by the originator of the test especially for formulations and the epidermal administration was recommended as a *"natural"* route, in contrast to exposure by intradermal injection. However, shaving *"till glistening of the application site"* and the pretreatment with dry ice for 10 seconds cannot be considered natural.

Although the method appears sensitive, as shown by positive reactions to benzocaine, it is not used in many laboratories and only a small number of published results is available (Maguire, 1975).

1.2.6. Buehler Test. This test was originally designed to detect moderate to strong sensitisers prior to testing in man. Although it is the second most widely used procedure (ECETOC,1980) a general concern is the existance of various modifications in test protocol which makes inter-comparison of data rather difficult. The sensitivity of the test has been questioned as negative results have been obtained with various human sensitisers, e.g. CPY, a pyrazolone derivative (Maurer, 1985). However, Robinson et al. (1989) have pointed out that some comparative studies reporting poor Buehler Test results have been flawed by the use of an improper test method. Practical experience has shown that

the Buehler Test, when properly performed, is able to detect moderate and weak sensitisers.

1.2.7. Open Epidermal Test (OET). Similar to the Buehler Test, the OET uses epidermal applications without adjuvant treatment. It is the only test in which an induction dose-response forms a part of the standard protocol. Four induction test groups are recommended, so this test requires more animals than the other methods.

1.3. Comparison of the Sensitivity and Reproducibility of the Test Methods

The main difficulty in comparing the sensitivity and reproducibility of different methods is that only a small number of compounds have been tested with several test methods (cf. Table 1). A good comparison can be made only for benzocaine, dihydrocoumarin, formalin, cinnamic aldehyde, PPD and penicillin G, which have been tested in the majority of the seven recommended test methods. The large variation in the incidences of sensitisation reflects the use of different induction concentrations and also variations in the way tests have been conducted in different laboratories.

The results in Table 1 again underline the importance of validating the test methods with clinically relevant mild/moderate allergens in each laboratory.

1.4. Relevance of Experimental Results to Man

A test in the guinea pig model can provide an indication only of a potential of a substance to cause skin sensitisation. As discussed above, different results may arise from the use of different methods and from interlaboratory differences. The purpose of this section is to indicate the ways in which these variable data can be related to man.

The basis of the interpretation is the test result (which might include dose response data) examined in the light of the sensitivity of the model to human contact allergens and the expected degree of human

exposure. Exposure may be influenced by several variables, viz. concentration, nature of the formulation, frequency, site and duration of exposure and the size of population likely to come into contact with the substance. Other considerations affecting interpretation include those relating to the penetrability of guinea pig skin (as compared with human skin) and the prevalence of damage to the barrier function of the skin (e.g. in hand eczema).

An important aspect of the guinea pig data is the relationship of response to the dose. With the exception of the OET, dose-response studies do not form a part of any standard protocol; they are of most value in assessing the relative sensitisation potential of classes of substances using an individual test. The relationship of animal dose/response data to man is unclear and may vary depending on, for example, whether induction or elicitation of sensitisation is being considered (Buehler and Ritz, 1985). For example, Kathon CG can sensitise the guinea pig and dose response studies have been carried out using the Buehler method (Chan et al., 1983); the minimum concentrations for induction and elicitation of 25 ppm and 100 ppm respectively can be directly compared with the figures of 12.5 ppm and 25 ppm obtained in a HRIPT (Cardin et al., 1986).

A more important but difficult objective is to establish a relationship between animal data and epidemiological findings. An understanding of this relationship for known contact allergens would aid assessment of the relevance to man of experimental animal data on a new chemicals. Numerous publications have assessed the skin sensitisation potential in the guinea pig of small groups of human contact sensitisers (Buehler, 1965; Magnusson and Kligman, 1970; Goodwin et al., 1981; Maurer, 1983). These studies show that, with very few exceptions, substances which have sensitised man can also sensitise the guinea pig. Some authors (Magnusson and Kligman, 1970) consider that a negative result in a sensitive guinea pig model does not prove absence of allergenic potential although Wahlberg and Boman (1985) have commented on the near impossibility of inducing contact allergy in guinea pigs with substances that have not caused allergy in man.

Some examples illustrate the above points. Isoeugenol is a flavour and perfume ingredient which is a sensitiser in the guinea pig (Maurer, 1985; Tsuchiya et al., 1985). Adjuvant tests using induction concentrations in the range of 0.1 - 10 % demonstrated that isoeugenol has a strong sensitisation potential. This suggests that unrestricted use of isoeugenol in perfumes, especially those designed for prolonged skin contact, would result in human sensitisation. Isoeugenol has sensitised man both in investigative procedures (Thompson et al., 1983) and from use of products containing it (Eiermann et al., 1982; Adams and Maibach, 1985; de Groot et al., 1985). Consequently the use of isoeugenol is subject to maximum recommended limits in perfumes/flavours (IFRA, 1984). The chemical is also included in the standard battery of fragrance allergens used in dermatological practice.

The relationship between animal data and the situation in man is more complex with nickel. Nickel is one of the commonest causes of allergic contact dermatitis (Cronin, 1980; Fisher, 1986; Schubert et al., 1987). Nickel, tested as nickel sulphate, is not a potent sensitiser in most guinea pig models, producing a moderate response in the Maximisation Test (Magnusson and Kligman, 1970; Goodwin et al., 1981) and no response at all in a less sensitive procedure such as the Draize Test (Johnson and Goodwin, 1985). The high incidence of clinical sensitisation in man may be due to the large proportion of the population who are exposed to nickel. It is ubiquitous and the constant exposure to it, e.g. in jewellery, would seem to increase the risk of populations becoming sensitised.

The paraben group are among the most widely used of preservatives. They are, at most, weakly sensitising in model systems (Goodwin et al., 1981) but because of extensive use at relatively high concentrations and in subjects with a compromised skin, they do sensitise man (de Groot, reviewed by Fisher, 1986). The relatively high test concentrations necessary to induce sensitisation in animals and the very low sensitisation potential led to the conclusion that human sensitisation to this group of preservatives would rarely arise. The clinical data have borne this out.

The sensitisation potential of a substance to man can be judged from test results in animals only by taking into account the likely human exposure. Evidence suggests that strong sensitisers may be used safely if there is a short period of contact to a low concentration and the skin is normal. Conversely, although test data may indicate a low sensitisation potential, sensitisation may occur in some individuals who have sufficient (repeated or prolonged) exposure.

2. *METHODS USING HUMAN VOLUNTEERS*

2.1. *General Comments*

Tests in human volunteers by-pass the one basic problem of all animal tests, that the test species may react differently from man. Depending on the method used, however, uncertainties can remain as to the relevance of the findings to the real life situation. The main question surrounding the use of skin sensitisation tests in human beings is that of their ethical acceptance. This has limited the use of such studies in many countries with, as a consequence, some reluctance by authorities to accept data generated by them, particularly in Europe. Unfamiliarity with the method, questions on their sensitivity and the fact that use of human test data was not foreseen by the existing EEC legislation may have contributed to this attitude.

There are no internationally agreed test protocols but several approaches are well supported by published data (Shelanski and Shelanski, 1953; Griffith, 1969; Stotts, 1980 and Kligman and Epstein, 1975). The way tests are conducted differs between researchers. This may be unavoidable but it complicates the interpretation and comparison of test results.

It is recommended that test methods using human volunteers should be standardised.

2.2. Specific Comments

2.2.1. Ethical considerations. Like other clinical experiments, sensitisation studies in human beings must follow the criteria identified in the rules of the Helsinki Agreement (Declaration of Helsinki, 1983). The risk of inducing contact sensitisation in volunteer panellists should be minimal (based on assessment of preclinical sensitisation data) and testing should be conducted on subjects who have been informed of the purposes and the potential risks of the test, and who have signed a consent statement (Robinson et al., 1989).

2.2.2. Selection of doses. Most methods give no clear indications for the selection of concentrations for induction and challenge. Excessive irritation is considered undesirable, not only for the well-being of the volunteers but also because it might mask weak reactions and interfere with the interpretation of the results. For these reasons it is desirable to determine the irritancy of substances before selecting a concentration suitable for both induction and challenge. Also important is the selection of the amount of material to be applied; this will be related to the size and type of the patch used. It is important to select a sufficiently large patch area (Buehler and Ritz, 1985) and a patch material which serves as a reservoir for the test substance during the application period. This is different from systems used for diagnostic tests, where the exposure is intended to be minimal.

2.2.3. Selection of the test population. The selection of volunteers for human sensitisation tests inevitably results in the use of a non-homogeneous group (compared to homogeneous groups of test animals). This necessitates use of a large number of subjects in each test to obtain a sufficient sensitivity; this also allows the opportunity to examine the variability within different groups of the human population. It also allows the selection of specific groups of volunteers to match the populations at risk. For ethical reasons, subjects who are at a higher risk or whose sensitivity is reduced

(e.g. by their use of certain medications such as immunosuppressive drugs) are excluded from such tests.

2.2.4. Evaluation of skin reactions. Skin responses are evaluated by subjective visual scoring of erythema, oedema and other changes of skin condition, e.g. formation of vesicles. Particularly with weak reactions, this may lead to different interpretations by different investigators. This is further complicated by the fact that the condition of the "normal" skin shows broader variations in a human population than in a group of animals.

The first step in the evaluation of a sensitisation test in man is confirmation of the nature of the response. It is critical to differentiate reliably between irritation and sensitisation responses. Normally it is assumed that any reaction involving oedema, papules and/or vesicles, generally in addition to erythema, indicates an allergic response. Further confirmation of this would be the observation of an increase in intensity from the first to the second reading (48 and 72/96 hours after application), and/or occurrence of a similar response at a second challenge patch at a different anatomical site. Any reactions must be compared to the non-specific irritation reaction noted with the same concentration of the test material. In contrast to guinea pig tests, the volunteers are normally used as their own controls. There are indications that, with some individuals and some test materials, irritation reactions are produced which are similar in appearance to allergic responses. It is therefore important to confirm that a reaction is reproducible before it is considered as an allergic response.

2.3. Comparison of the Sensitivity of Different Test Methods

Skin sensitisation tests in man can be used both to clarify the sensitisation potential of a material and to examine the relevance of such a potential to real life situations. Therefore, the question of the relative sensitivity of the various methods cannot be answered in a general way; sensitivity depends rather on the specific purpose of a

test. Nevertheless, increasing the intensity of the exposure by increasing, for example, the number of applications or the level of occlusion, or by pre-treating the skin to enhance penetration of the test material will improve the sensitivity of methods.

2.4. Relevance of Human Results to Classification and Risk Assessment

When an allergic response has been confirmed, the sensitisation potential must be judged against expected exposure. In most instances, the exposure in a human sensitisation patch test is exaggerated compared to the real life exposure because of the concentration of the material, the occlusive nature of the patch and the duration of each exposure (24 hours in most tests). In addition, it must be recognised that the potential of a material to induce or elicit an allergic response depends on the dose per unit area of skin rather than the total dose applied (White et al., 1986). The fact that in normal life the compound may come into contact with a larger area of the skin is important only insofar as this may involve contact with skin areas which are more readily penetrated.

It is thus essential to estimate the real life exposure in terms of concentration, frequency and duration when performing a risk assessment for a specific use of a material. In addition, it may be possible to confirm by appropriate in-use testing whether reactions seen under patch test conditions are likely to occur when a substance or product is used normally. This may be done by observing reactions following open application of the test substance or a preparation made from it, supervised home use or workplace exposure.

Similarly, it is possible to combine controlled use with diagnostic patch tests to determine if longer term exposure to low concentrations of a suspected sensitiser is likely to induce allergy.

Human sensitisation tests have generally not been used for the classification of substances as non-sensitisers. This is because of an absence of internationally agreed test protocols, the difficulties in

including positive controls and because the methods for establishing the sensitivity of human tests are less developed than for animal tests. Nevertheless for products for which direct human contact is intended, predictive tests in human volunteers can be considered. Once sensitisation potential has been established in a suitable model, human tests can be of value in determining the relevance of that potential to man in specific use situations.

F. USE OF SKIN SENSITISATION TESTS FOR REGULATORY PURPOSES

The EEC (1984), US-EPA FIFRA/(1984)/TSCA (1985) and Japan/MAFF (1985) prescribe tests which are used for classification and labelling and which thus give an indication of a sensitisation potential but not the risk to human health. The EEC (1983) classifies sensitisers as harmful, requires them to be labelled with the St Andrews cross and imposes the Risk Phrase R 43 *"may cause sensitisation by skin contact"*. A substance is classified as a sensitiser if practical experience shows that substances and formulations induce sensitisation in a significant number of persons due to skin contact, or if positive results are obtained in experimental studies. A result is considered positive when, in an adjuvant method, 30 % or more of the test animals yield positive responses in the absence of reactions in the control animals. Occurrence of 15% positive responses or more in a non-adjuvant test method also leads to classification.

ECETOC agrees that this is a reasonable approach to classification and labelling of skin sensitisers. As indicated in Chapter E, certain existing test methods are preferred for the evaluation of skin sensitisation potential. Where a new test method is employed, the user should justify the choice and supply evidence of the validity of the procedure using mild/moderate human contact allergens.

Various methods are available for the determination of the percentage positive response. For example, calculation of the percentage of test animals which have a greater intensity or duration of response than the maximum reaction seen in the control animals. Alternatively, calculation of the percentage of control animals which have responded and subtraction of this from the percentage of responding test animals.

ECETOC agrees with the approach recommended by Shillaker et al. (1989), who suggest that, using these two methods, the analysis giving the higher result should be employed if the data are on the borderline of classification.

In the EEC Dangerous Preparations Directive (EEC, 1988) preparations containing 1% or more of a sensitiser are to be labelled unless a test on the preparation itself proves the contrary.

The choice of 1% was arbitrary; for strong sensitisers 1% may be too high a concentration, whilst for weak sensitising materials it may be too low, and it fails to take into consideration the likely human exposure. It should be possible to overcome this difficulty by, for example, testing the preparation itself or performing a dose-response study on the sensitising ingredient.

The Occupational Safety and Health Administration (OSHA-USA, 1987) in their *"hazard communication rule"* defines a sensitiser as *"a chemical that causes a substantial proportion of exposed people or animals to develop an allergic reaction in normal tissue after repeated exposure to the chemical"*. This definition is in line with the American National Standards Institute definition of a *"strong sensitiser"*. No equivalent to the EEC R-phrase exists. The EPA and Japan/MAFF do not specify criteria for labelling or classification of sensitisers.

G. ALTERNATIVE APPROACHES TO THE ASSESSMENT OF SKIN SENSITISATION POTENTIAL

During the last thirty years, significant advances have been made in our understanding of the mammalian immune system. In marked contrast, predictive tests for contact sensitisation potential have altered little since the introduction of the Draize Test in 1959. Appendix 2 and Appendix 3 list the many different guinea pig test methods which have been published since that time. As mentioned previously (cf. Chapter C), only the seven tests shown in Appendix 3 have become accepted worldwide by regulatory authorities. Several of the tests shown in Appendix 2 are still in use but often only in a few laboratories where they are used for specific purposes (e.g. as pre-screens for strong sensitisers, or in research studies).

This chapter describes some of the ways in which the currently accepted methods could be modified to improve the capacity to predict sensitisation potential, to expedite the process of risk assessment using the test data, to reduce the number of animals required and to minimise the occurrence of severe effects. Suggestions are also made for modifications which could improve the consistency of response in individual tests in different laboratories.

The recommended test methods are, without exception, empirical in their design and subjective in their means of assessment. With the increasing understanding of cellular immunology in general, and of immune responses in contact sensitivity in particular, there is an opportunity to develop further more objective and immunologically based predictive test methods.

One of the most stimulating challenges is to define the way in which the immune response to contact sensitising chemicals determines the sensitising potency of the chemical. Guidelines for the conduct of the guinea pig tests methods referred to in Chapter A do not require an assessment of the dose of a material below which it is impossible either to induce contact sensitisation or to elicit a dermatitic reaction in a previously sensitised animal. These assessments are in any case difficult to perform, often needing large numbers of animals and an extended testing period.

Risk assessment for contact sensitisation would be improved considerably by the introduction of practical and reliable methods which are capable of determining the potency of a sensitiser, based on dose-response.

This chapter describes a number of newly developed mouse tests for skin sensitisation potential, some of which appear to offer a practical approach to the determination of potency. A strategy for accelerating the development and validation of these tests can be found in Chapter H.

1. MODIFICATION OF EXISTING TECHNIQUES

Oliver et al. (1986) considered that the existing methods could be improved by shortening experimental protocols and reducing their complexity and cost without loss of sensitivity, and by introducing a clearer definition between irritation and sensitisation responses and a reduction of the number of animals used.

1.1. Reduction in the Number of Animals Used in Sensitisation Tests

There is considerable pressure to reduce the numbers of animals used both in test and control groups and some specific proposals have been made (Hofmann et al., 1987; Schlede et al., 1987; Shillaker et al., 1989). It is of great importance that the quality of the data produced in an animal model is not compromised to the point where its ability to assess hazard becomes uncertain. Particularly with adjuvant tests, some groups already use fewer animals than recommended by regulatory authorities.

Shillaker et al. (1989) have shown that it is now possible to obtain reliable results using as few as 10 test and 5 control animals for adjuvant test methods. Acceptance of this by a regulatory authority will depend critically on the quality of data presented for relevant positive controls (see below). Whilst a similar argument may be applied, in time, to non-adjuvant methods, at present there are no data to support an immediate reduction in the number of animals required for these tests.

1.2. Reference Substances

It has become common practice for a positive control substance to be tested every six months and for a summary of the results to be submitted alongside the data for a new substance. This allows a check to be made on the sensitivity of both the test method and the guinea pigs used by the particular laboratory, and is recommended as good practice.

Since positive control data will rank the sensitivity of the method employed, extreme sensitisers such as DNCB and PPD should not be used as positive controls. Instead, mild or moderate sensitisers, such as benzocaine, neomycin sulphate, hydroxycitronellal or any other chemical known from the literature to be a mild to moderate sensitiser should be used. To justify the use of fewer animals in an adjuvant test, more than 20% positive responses should be obtained with these materials.

1.3. Rechallenge

When there is an inconclusive result at challenge or when positive reactions are seen in control animals, consideration should be given to re-challenge, perhaps with a lower dose. The interpretation of the incidence of sensitisation from multiple challenge data must be determined on a case by case basis.

1.3. Histopathology

Maurer (1983) reviewed the evidence for distiguishing between allergic and irritant reactions using histopathology; although several publications have indicated that, for example, more polymorphonuclear cells are seen in irritant reactions, others have shown that there are no clear-cut differences. Since it is difficult to differentiate reliably between the two types of reactions histologically, identification of a sensitisation response is possible only when the inflammatory response is significantly more marked in the test animals than in the controls.

2. *NEW IN VIVO TESTS*

For more than 40 years the guinea pig has been the animal of choice for predictive sensitisation tests and it is still the only animal species accepted by the authorities.

The immune system of the mouse has been investigated more extensively than that of the guinea pig. It should, however, be noted that marked differences in sensitivity to contact allergens have been observed in various inbred mouse strains (Asherson and Ptak, 1968; Dietrich and Hess, 1970; Shultz and Bailey, 1975; Corsini et al., 1979; Bäck and Larsen, 1982; Sauder and Katz, 1983).

New tests using the mouse as experimental animal have recently been published. Many of the tests include a topical induction treatment on the mouse abdomen and a challenge application on the ear (Gad et al., 1986; Maisey and Miller, 1986; Cornacoff et al., 1988; Descotes, 1988). In all these experiments, objective parameters for the measurement of the allergic reaction were used, most commonly by assessing an increase in ear thickness. Nevertheless, to obtain reproducible results using this technique, extensive experience is required and the animals are often anaesthetised.

Alternative methods for evaluating the challenge reaction have included the measurement of the wet weight increase of treated ears (Corsini et al., 1979; Möller, 1984; Chapman et al., 1986) and an assessment of the cellular infiltrate using radioactive compounds (Bäck and Larsen, 1982; Chapman et al., 1986; Cornacoff et al., 1988). Another method is based on histomorphometric evaluations; promising results were obtained by Bratanow et al. (1981) using this technique.

Dose-response studies for induction and challenge were performed by Dietrich and Hess, 1970; Sy et al., 1977; Bäck and Larsen, 1982 and Thorne et al., 1987. Sy et al. (1977) found that the optimal sensitising dose is not necessarily the highest dose applied; they observed active

immunosuppression with supra-optimal sensitising doses of dinitrofluorobenzene (DNFB).

Most of the experiments on cellular mechanisms and methods in delayed hypersensitivity have used strong allergens such as oxazolone (Dietrich and Hess, 1970; Bäck and Larsen, 1982; Shultz and Bailey, 1983; Chapman et al., 1986), DNFB (Corsini et al., 1979) or isocyanates (Thorne et al., 1987). Experience with weak allergens in predictive mouse protocols has been addressed in two recently published methods.

Maisey and Miller (1986) tested the contact sensitising potential of various compounds in mice maintained on a diet supplemented with vitamin A acetate. They measured the increase of mouse ear thickness after epicutaneous induction and challenge. Weaker human sensitisers, such as formaldehyde, benzocaine, citral and dihydrocoumarin produced positive responses.

Gad et al. (1986) published the mouse ear swelling test (MEST) and obtained promising results with 50 test compounds. Cornacoff et al. (1988) compared the MEST and a radioisotopic incorporation method and found that the mouse was unable to detect weak sensitisers such as nickel sulphate.

Another promising method monitors the primary T-lymphocyte response in the draining lymph node following topical application to the mouse ear (Kimber et al., 1986, 1987; Kimber and Weisenberger, 1989). This method has the advantages of a short duration, an objective measurement of proliferation and minimal animal treatment.

3. *IN VITRO TESTS*

Since contact allergy is a multicomponent reaction involving several organ systems (skin, lymphatics and blood), it has not been possible to develop a practical in vitro model, although attempts with very strongly sensitising chemicals have met with limited success (Parish, 1986; Hauser and Katz, 1988). Individual components of the reaction can be assessed in

vitro: for example skin penetration (e.g. from the partition coefficient) and reactivity with protein or a standard nucleophile (e.g. serum albumin or butylamine). Nevertheless, the relationship between these individual components of the response and the ability of the chemical to behave as a sensitiser is not clear.

4. *USE OF QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIPS (QSAR)*

QSAR represent the attempt to predict biological activity from the structural and physico-chemical characteristics of chemicals. In some areas of toxicology, QSARs have been found which show some promise. Where the biological activity is a simple toxic phenomenon related to concentration, the main determinant is often the octanol/water partition coefficient even for varying families of chemicals (Turner et al., 1987). For contact sensitisation, the number of variables is considerably greater. A mathematical model of contact allergy in the guinea pig was described by Roberts and Williams (1982). This failed, however, to take account of separate induction and challenge doses, intrinsic antigenicity of chemicals, cutaneous biotransformation and antigen specific tolerance. Some of these aspects are considered in a revised description (Roberts and Basketter, 1989), but the model still can be applied only to individual families of chemicals. Thus, whilst the QSAR approach may represent the most likely route to a non-animal model, this goal is still a long way off.

H. STRATEGY FOR ACCELERATING THE DEVELOPMENT, VALIDATION AND USE OF ALTERNATIVE TEST PROCEDURES

Chapter G described the background to the use of the mouse in immunological investigations of skin sensitisation. The knowledge gained from some of these fundamental studies has led to the development of several models of skin sensitisation in the mouse.

Two models, the Mouse Ear Swelling Test (MEST) and the Local Lymph Node Assay (LLNA), have shown promise. It is recommended that further inter-laboratory testing should be performed to determine their sensitivity and specificity in comparison with the accepted guinea pigs tests and to investigate their robustness. The two models and their major features are summarised in Table 2.

Although several inter-laboratory trials have been conducted (e.g. in the USA with the MEST, Gad et al., 1986), or are in the process of being completed (e.g. the LLNA is currently under investigation in four laboratories in the UK), it is important that the relative strengths and weaknesses of the models are fully assessed by means of more extensive comparisons conducted in a number of laboratories across the world.

The organisation of these comparative studies could be co-ordinated by an international organisation such as the OECD or WHO-IPCS.

Until such investigations have been completed, alternative models should not replace the currently accepted guinea pig tests for classifying and labelling sensitisers, unless the data demonstrate that the substance consequently should be classified as a sensitiser.

TABLE I

SENSITISATION POTENTIAL OF A RANGE OF CHEMICALS IN SEVERAL TEST PROCEDURES

Test Chemical	Test Method*						
	M & K	Buehler	Optimisation	SAT	FCAT	OET	Draize
DNCB	100	100	100	-	-	-	100
Benzocaine	0 - 85	20	0 - 90	40 - 85	40 - 90	40 - 60	0
Cinnamic aldehyde	60 - 100	0 - 50	100	100	-	100	10 - 20
Dihydrocoumarin	100	100	100	100	100	88 - 100	100
Dowicil	55 - 90	0	-	37	-	-	0
Ethylene diamine	90 - 100	-	90	40	-	-	50
Eugenol	0 - 60	0	30	-	-	-	-
Formalin	18 - 100	30 - 60	10 - 100	0 - 70	20 - 60	0 - 50	10 - 70
Germall 115	10 - 70	0	0	0	-	-	0
Hydroquinone	70	-	-	-	-	-	30
Hydroxycitronellal	27 - 30	0 - 25	-	17	-	-	0
Isoeugenol	70 - 100	-	100	-	100	100	-
Mercuric chloride	32	-	-	-	-	-	0
Neomycin	30 - 90	-	55	-	-	25 - 45	0
Nickel	10 - 60	0	100	-	-	-	0
Paraphenylene diamine	10 - 100	100	5 - 100	100	-	-	0 - 100
Penicillin G	70 - 100	-	33 - 100	80 - 100	67 - 100	0 - 75	35
Potassium dichromate	10 - 100	10	95	-	-	-	10 - 20
Tetrachlorosalicylanilide	72 - 88	80	100	-	-	-	0
Methyl methacrylate	0 - 77	-	-	0	-	-	-

* Results expressed as the minimum-maximum percentage rate of sensitisation for that chemical and method found in the literature.

\- not tested

TABLE 2

COMPARISON OF TWO ALTERNATIVE TEST PROCEDURES

TEST	INDUCTION APPLICATION	CHALLENGE APPLICATION	CRITERIA FOR POSITIVE REACTION
Mouse Ear Swelling test (MEST) (Gad et al., 1986)	1) (a) Adjuvant injection (b) Tape stripping of application site (c) Epidermal (flank) (x1) 2) (a) Tape stripping of application site (b) epidermal (flank) (X3)	Epidermal	> 20% increase in ear thickness above that of vehicle-treated control ear.
Local lymph Node Assay (LLNA) Kimber et al., (1989)	Epidermal (both ears) (X3)		> 3-fold increase in lymphocyte proljferation in draining lymph node of test animals compared with vehicle-treated control mice.

Applications are unoccluded in the two tests.

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APPENDICES

APPENDIX 1

GLOSSARY OF TERMS

Adjuvant : a substance used to enhance the immune response in a non-specific manner, such as Freund's Complete Adjuvant.

Allergy : a clinical manifestation of a state of hypersensitivity. Allergy is defined classically as an antigen specific altered reactivity of the host to antigen.

Antigen : foreign material which can induce an immune response (see also Hapten).

Antigen presenting cells : a number of cell types which concentrate, process and present antigens to lymphocytes in order to induce an immune response.

Carrier protein : the protein with which the hapten reacts to form antigens.

Cell-mediated immunity : an immune response mediated by antigen specific lymphocytes.

Challenge : treatment designed to elicit a skin reaction in a sensitised animal/man.

Dendritic cell : see antigen presenting cells.

Erythema : a reddening reaction in skin caused by a local increase in peripheral blood flow.

Hapten : a small molecule, which although foreign, cannot be recognised by the immune system unless combined with a suitable carrier (which is believed to be a protein, cf. Carrier protein).

Hazard : a toxic effect, in this context a sensitising effect occurring as a consequence of exposure.

Hypersensitivity : an adaptive immune response against an antigen which occurs in an exaggerated or inappropriate form and which can lead to tissue damage (see allergy).

T-Lymphocytes : cells of bone marrow origin which mature in the thymus and then migrate into blood, lymph and lymphoid tissue. They express antigen receptors and are divided functionally into helper and suppressor/cytotoxic subpopulations.

Lymphokine : generic term for a number of molecules (excluding antibody) produced by lymphocytes and involved in mediating cellular interactions during an immune response.

Macrophage : bone marrow derived mononuclear cell found in the blood (where it is known as the monocyte), lymph and in many organs. Two main functions are recognised, phagocytosis and antigen presentation (cf. antigen presenting cell).

Memory cells : circulating antigen specific T lymphocytes which arise following sensitisation by clonal expansion.

Necrosis : tissue death.

Oedema : tissue swelling caused by movement of fluid from the vasculature to the tissue.

Risk : the probability of a hazard being expressed.

Sensitisation : the development of an expanded population of T-lymphocytes for a specific antigen which can give rise to a delayed allergic response upon challenge with that antigen.

APPENDIX 2

ADDITIONAL GUINEA PIG SENSITISIATION METHODS

Modified Draize Test	(Voss, 1958)
Epidermal Allergenicity Test	(Weirich, 1962)
Stevens Test	(Stevens, 1967)
TINA-Test	(Ziegler, 1976, 1977)
Modified Split Adjuvant Test	(Prince and Prince, 1977)
Modified Draize Test	(Sharp, 1978)
Single Injection Adjuvant Test	(Goodwin et al., 1981)
Footpad Test	(in OECD, 1981)
Modified Guinea Pig Maximization Test	(Sato et al., 1981)
Cumulative Contact Enhancement Test	(Tsuchiya et al., 1982)
Epicutanous Maximization Test	(Guillot et al., 1983; Guillot and Gonnet, 1985)
Guinea Pig Allergy Test	(Dossou et al., 1985)
Modified Maximisation Test	(Maurer and Hess, 1989)

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APPENDIX 3

SPECIFIC FEATURES OF OECD STANDARD TESTS

The summaries below give only the principles of each method and should not replace the reference to the original publications. The data in the review below are based on the original publications and may diverge from the overview published in the OECD Test Guideline.

1. DRAIZE TEST (Draize et al. 1944, Draize 1959)

■ ■ ■ ■ ■ ■ ■ ■ ■
/...../...../...../...../...../...../...../...../
1 8 15 22 36 Day

■ = intracutaneous injection

Number of animals: test group up to 20 animals per group.

Control group not specified.

Test concentrations for induction and challenge: 0.1 % solution or suspension of test substance in physiological saline.

Test procedure:

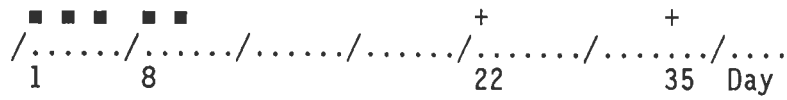
Induction: ten intracutaneous injections are made on the back of the animals. The injections are made every other day or three times weekly. For the first injection, a volume of 0.05 ml is used; for injections 2 - 10 a volume of 0.1 ml is required.

Challenge: two weeks after the last induction application, a challenge injection (0.05 ml) is made on the flank.

Evaluation of reactions: 24 hrs after each induction application the diameter, elevation and colour of reactions are read. The challenge reaction is compared with the average score of the induction reactions. No scoring scale or exact measurement system for the evaluation of the reactions is described.

Criteria for positive reaction: the challenge reaction score is compared to the average score observed during induction to determine whether the substance has produced sensitisation.

2. FREUND'S COMPLETE ADJUVANT TEST (Klecak et al., 1977)



■ = intracutaneous injections
+ = epidermal open application

Number of animals: test and control up to 20 animals per group.

Test concentrations:

Induction: 5% in FCA / distilled water (1/1).

Challenge: 4 concentrations; the highest concentration should be minimally irritant. The other concentrations should be successive threefold dilutions.

Test procedure:

Induction: 0.1 ml of the test compound as an FCA suspension is injected every second day in the shoulder region of the animals (5 injections in total).

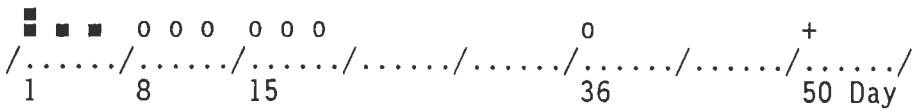
Challenge: 4 concentrations are applied on the flank on day 22 and 35. Each concentration (0.025 ml) is applied in an area of 2 cm^2.

Control group: the control group is treated with FCA alone during the induction and with the 4 test compound concentrations during the challenge.

Evaluation of reactions: the erythema, oedema and scaling formation is graded 24, 48 and 72 hrs after challenge application.

Criteria for positive reaction: "The test material is considered allergenic when animals of the experimental group show positive reactions to nonirritant concentrations used for challenge."

3. OPTIMISATION TEST (Maurer et al., 1978, 1979; Maurer, 1983)



■ = intracutaneous injections
o = intracutaneous injections with adjuvant
+ = epidermal occlusive application

Number of animals: test and control group up to 20 animals per group.

Test concentrations:

Induction: 0.1 % solution or suspension of the test material in physiological saline or another appropriate vehicle.

Challenge: for the intradermal challenge the same solution or suspension as during the first week of induction is used. The epidermal challenge is made with the maximal sub-irritant concentration of the test compound in petrolatum.

Test procedure:

Induction: during the induction period the animals receive one injection into the skin three times a week for 3 weeks. During the first week the injections are made into the right flank and midback skin (first day two injections). During the 2nd and 3rd week the test compound, suspended in FCA, is injected into the nuchal skin.

Challenge: a single intradermal challenge injection is made into the left flank. An epidermal occlusive challenge application is made 10 days after the intradermal challenge injection.

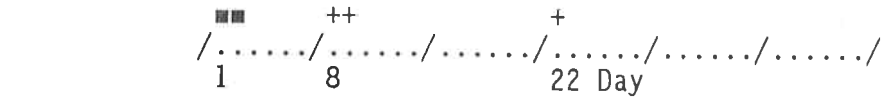
Control group: the control group is treated with the vehicles and FCA.

Evaluation of reactions: during the first induction week and following the intradermal challenge application the erythema reaction diameter and the skin fold thickness increase are measured 24 hr after injection and an approximate reaction volume is calculated for each individual animal. The reaction volumes of induction and challenge are compared for each animal.

The challenge reactions after epidermal application are evaluated 24 hrs after removing the bandages according to the Draize scoring scale for primary skin irritation (Draize, 1959).

Criteria for positive reaction: the number of responding animals in the test group is compared statistically (using the Fisher exact test) with the number of responding animals in the control group treated with the vehicle alone. The comparison is made separately for the results obtained after intradermal and epidermal challenge. A threshold of significance of $p < 0.01$ is used (Maurer, 1974).

4. *MAXIMISATION TEST* (Magnusson and Kligman 1969, 1970; Magnusson, 1980)



■■ = 3 pairs of intracutaneous injections
++ = epidermal occlusive application, 48 hrs
+ = epidermal occlusive application, 24 hrs.

Number of animals: test and control up to 25 animals per group.

Test concentrations:

Induction: for intradermal application the concentration chosen must be as high as possible without producing general toxicity. If the substance is skin irritating the concentration chosen for epidermal application should elicit slight to moderate irritation. If the substance is non-irritant the

site of application is treated with 10% lauryl sulphate in petrolatum 24 hrs before the topical induction.

Challenge: maximal sub-irritant concentration.

Test procedure:

Induction: three pairs of intracutaneous injections are made into the nuchal skin on day 1 with

- two injections with the test compound in saline or another appropriate vehicle,
- two injections with an adjuvant/water suspension,
- two injections with the test compound in the adjuvant/water suspension.

One week later the test compound is applied neat or incorporated in a vehicle and applied to the injection sites on the animals neck epicutaneously under occlusion for 48 hrs.

Challenge: 2 weeks after the epidermal induction application, the animals are tested on the flank with the maximal sub-irritant concentration.

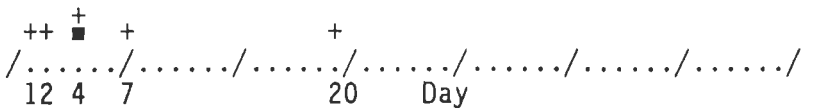
Control group: a control group is treated with the vehicle and FCA during the induction period. The animals are challenged in the same way as the test animals.

Evaluation of reactions: the reactions are graded according to a four point scale 24 and 48 hours after removing the dressings.

Criteria for positive reaction: the sensitisation potential of the test compound is classified according to the frequency of positive responses in test and control animals (cf. p. 29).

sensitisation rate	grade	classification
0-8 %	I	weak
9-28 %	II	mild
29-64 %	III	moderate
65-80 %	IV	strong
81-100 %	V	extreme

5. *SPLIT ADJUVANT TECHNIQUE* (Maguire and Chase, 1972; Maguire, 1973, 1975)



\+ = epidermal occlusive application
■+ = adjuvant injection and epidermal occlusive application

Number of animals: test and control up to 20 animals per group.

Test concentrations:

Induction: "use concentration"

Challenge: sub-irritant concentration of the final formulation or test solution.

Test procedure:

Induction: the animals are covered with a dressing fitted with an open window of 2 x 2 cm below the right side of the shoulder girdle. Test solutions are applied on filter paper patches on day 1 and 2 for 24 hours and for 48 hours on day 4 and 7. Two adjuvant injections are made before the epidermal application on day 4.

Challenge: a single 24 hrs epidermal occlusive application is made on day 20.

Control group: treatment not specified in publications.

Evaluation of reactions: the reactions are graded according to a 7 point scale directly after removing the challenge dressings and 24 and 48 hrs later.

Criteria for positive reaction: not specified in publications.

6. *BUEHLER TEST* (Buehler, 1965; Buehler and Griffith, 1975; Ritz and Buehler, 1980; Robinson et al., 1989)

```
 +      +      +             +            (+)
/....../....../......./......./......./......./......./
 1      8      15            29            43  Day
```

\+ = epidermal occlusive application for 6 hours.
(+) = rechallenge (if needed)

Number of animals: up to 20 animals per group, 10 per control group.

Test concentrations:

Induction: minimal irritant concentration.

Challenge: maximal sub-irritant concentration.

Test procedure:

Induction: the test compound is applied in a suitable vehicle under tightly occluded patches to the preshaven left shoulder. The animals are kept in restrainers during each application of 6 hours.

Challenge: a challenge patch is applied to a naive skin site on the flank of the animal.

Control group: a naive control group is challenged in the same way as the test group; use of control group treated with the vehicle only may be considered.

Evaluation of reactions: the reactions are graded according to a 5 point scale (0, ±, 1,2,3) 24 and 48 hours after removing the patches.

Criteria for positive reaction: skin grades of 1, 2 and 3 are considered positive. The decision whether a test compound is a sensitiser is based on comparisons of results (incidence and severity) in test and control group(s).

7. OPEN EPIDERMAL TEST (Klecak et al., 1977)

```
 ++++++++++++++++++++                +
/...../...../...../...../...../...../...../
 1      8      15     22            36 Day
```

+ = epidermal open application

Number of animals: up to 6 experimental groups with up to 8 animals per group.

Test concentrations:

Induction: depending on the irritancy of the test compound, 100%, and dilutions of this concentration in steps of 3 applied to an area of 8 cm^2.

Challenge: 4 concentrations; the highest concentration should be minimal irritant. The other concentrations should be successive threefold dilutions. They are applied individually on an area of 2 cm^2.

Test procedure:

Induction: the solution is applied on 21 consecutive days or 5 times per week during 4 weeks on the right flank of the animals.

Challenge: challenges are performed on day 22 and on day 36 on the left flank.

Control group: a control group may be treated with the vehicle alone during the induction. The same 4 test compound concentrations as in the test group are applied for the challenge application.

Evaluation of reactions: the challenge reactions are graded 24, 48 and 72 hrs after the challenge application.

Criteria for positive reaction: the minimal sensitising and minimal eliciting concentration is determined.

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APPENDIX 4

MEMBERS OF THE TASK FORCE

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D.A. BASKETTER	UNILEVER RESEARCH UK - Sharnbrook
T. MAURER	CIBA - GEIGY CH - Basel
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