4. Skin Sensitisation

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1. Current data requirements and current tests

Rules related to the safety of cosmetic products are imposed by the Council Directive 76/768/EEC (Cosmetics Directive) of 27 July 1976 (with subsequent amendments and adaptations). Article 2 states that: "A cosmetic product put on the market within the Community must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use ..." Article 7 explains that "... the manufacturer shall take into consideration the general toxicological profile of the ingredient, its chemical structure and its level of exposure."

In order to help manufacturers in complying with this legal obligation, the Commission's Scientific Committee on Cosmetics and Non-Food Products (SCCNFP) issued a guidance document (SCCNFP, 2000) that has the purpose to provide guidance for testing cosmetic ingredients and for the safety assessment of the finished product, both to the competent monitoring authorities of the Member States, and to persons responsible for putting cosmetics on the market (manufacturers or importers within the European Union).

1.1 For cosmetic ingredients

In general, the regulations of the Council Directive 67/548/EEC on the classification, packaging and labelling of hazardous substances apply also to cosmetic ingredients. Some ingredients, e.g. certain plant extracts, do not fall under the directive.

Although the SCCNFP pursues the aim of replacing in vivo sensitisation tests with in vitro alternatives, it conceded that no validated alternatives are available to date (SCCNFP, 2000). It therefore requires cosmetic ingredients to be tested in an animal test according to OECD and EU test guidelines and according to Good Laboratory Practice (GLP).

The SCCNFP has evaluated the use of human tests for assessing the sensitisation hazard and concluded that: "As a general rule, cosmetic ingredients identified as sensitisers in animal assays or other validated assays when existing should not be studied in humans. The human sensitisation tests are time consuming and very expensive because a large number of volunteers is required in each test (150-200), though, considerable less for the human maximisation test (25) which, as the name says, maximises the response to a certain degree. Further, the selection of human volunteers usually results in the use of an inhomogeneous test group (compared with the more homogeneous group used for animal experiments). The large numbers of participants in most of these tests are necessary in order to reduce the 95% confidence interval for the test result, otherwise the likelihood of unpredicted responses in the consumers increases. If, for instance, no positive reaction occurred in 100 induced test subjects, for statistical reasons up to 36 of 1000 consumers may react. The argument for reducing the number of volunteers in the human maximisation test is the amplifying step
introduced by treatment with an irritant test product or sodium lauryl sulphate. In any case, it is scientifically inadequate and unethical to perform predictive tests with a number of subjects insufficient to produce valid data."

It explains that: "Predictive human sensitisation tests involve attempts to induce a long lasting or permanent immunologic sensitisation in the individual. Therefore, serious ethical questions arise. In spite of many years of experience with human sensitisation tests, very limited scientific information is available in the literature regarding the consequences involved for the human volunteers who have developed a patch test sensitisation during such a test." and concludes that: "Due to the uncertainties mentioned above it is the opinion of the SCCNFP that predictive human sensitisation tests of potentially cutaneous sensitising cosmetic ingredients or mixtures of ingredients should not be carried out without a better understanding of the immunologic background and mechanisms underlying positive reactions in these kind of tests in human beings. Further, it is questionable whether predictive testing on humans contributes to human safety in comparison with animal testing. At the present, no alternative method for predicting sensitisation has been validated." (SCCNFP, 2000).

1.2 For cosmetic finished products

Finished cosmetic products are exempt from Directive 1999/45/EG on the classification, packaging and labelling of hazardous preparations.

Since several years, the cosmetic industry carries out the safety evaluations of finished cosmetic products through the use of animal tests on single ingredients, existing data, historic databases and computer expert systems. Finished cosmetic products are not tested on animals any more.

With regard to sensitisation testing in human subjects, see opinion of SCCNFP in Section 1.1.

1.3 Available animal tests

In the following, those animal tests are reported, for which test guidelines have been developed. Besides these, other protocols for sensitisation tests in guinea pigs (e.g., Draize test, open epicutaneous test, optimisation test, split adjuvant test) are available.

1.3.1 Mouse Local Lymph Node Assay (LLNA)

1.3.1.1 Short description, scientific relevance and purpose

In the LLNA, the test substance is applied onto the mouse ear on three consecutive days. On the sixth day, the proliferation of lymphocytes in the draining lymph node, caused by the primary immune reaction, is measured. This is done usually by measurement of the incorporation of ³H-methyl thymidine into the DNA of proliferating lymphocytes in draining lymph nodes. The LLNA does not include a challenge phase. The endpoint of interest is the stimulation index giving the ratio of thymidine incorporation in lymph nodes from dosed animals compared to the incorporation in lymph nodes from vehicle-treated control animals. The test is positive when the stimulation index $\geq 3$ (SI ≥3).

The LLNA has been accepted by the Interagency Co-ordinating Committee on the Validation of Alternative Methods (ICCVAM) in USA as a stand alone alternative to the current guinea pig tests, and as an improvement for animal welfare because it reduces and refines animal use in the hazard identification of skin sensitising substances. The LLNA has been recognised as a
standalone test for skin sensitisation by competent authorities of European Union member states and by the SCCNFP.

Another advantage of the LLNA over the guinea pig assays is that the EC3 value, which is the effective concentration of the test substance (percent of substance in vehicle) required to produce a threefold increase in the stimulation index compared to vehicle-treated controls can be used as a quantitative measure of the sensitising potency of the test substance. This might form the basis for a quantitative risk assessment for skin sensitisation in the future.

1.3.1.2 References

Kimber et al., 1986; 1989; Kimber et al., 2002
ICCVAM evaluation: Dean et al., 2001; Haneke et al., 2001; Sailstadt et al., 2001

1.3.1.3 Status of validation and/or standardisation

A test guideline is available as OECD Test Guideline No. 429.

1.3.2 Mouse Ear Swelling Test (MEST)

1.3.2.1 Short description, scientific relevance and purpose

The MEST comprises both the induction phase and the elicitation phase of the immune response. Several weeks prior to and during the test period mice are fed a diet enriched in vitamin A since this has been shown to enhance contact sensitisation. In the protocol which has been used most frequently the induction phase comprises clipping of the fur on the belly region and removal of the outer layers of the epidermis by tape stripping. Freund's Complete Adjuvant (FCA) is injected intradermally before the test substance in vehicle (test mice) or vehicle alone (control mice) is applied topically. The skin is tape stripped each day during the following four days and on days 1, 3 and 5 after FCA injection the same amounts of test substance or vehicle alone are again applied topically. After a rest period of five days the challenge phase begins on day 10 with topical application of test substance at the maximum non-irritating concentration to one ear and of vehicle alone to the other ear of all (test and control) mice. Ear thickness of test and control ears is measured under ether anaesthesia with a micrometer 24 and 48 hours after application of test substance. Ear swelling is expressed as the difference between test and control ears in percent.

The MEST has been evaluated independently by several laboratories and in inter-laboratory studies. It was concluded that the MEST is a useful model for identifying strong contact sensitisers.

The Mouse Ear Swelling Assay (MESA) is a variant of the MEST with some modifications in the test protocol. The use of the MESA is very limited.

1.3.1.2 References

Asherson and Ptak, 1968; Gad, 1986; 1994
Evaluation: Cornacoff et al., 1988; Descotes, 1988; Dunn et al., 1990; Gad et al., 1987
(Cornacoff et al., 1988; Hignet et al., 1989; Dunn et al., 1990)

1.3.1.3 Status of validation and/or standardisation

No standardised test guideline is available for the MEST.
1.3.3 Magnusson Kligman Guinea Pig Maximisation Test (GPMT)

1.3.3.1 Short description, scientific relevance and purpose

The GPMT is a highly sensitive method using Freund’s complete adjuvant as an immune enhancer. It includes both intradermal and topical induction treatment and closed challenge. Three pairs of intradermal injections are given in the shoulder region which is cleared of hair: injection 1 is a 1:1 mixture of Freund's complete adjuvant and physiological saline, injection 2 is the test substance at the selected concentration in an appropriate vehicle and injection 3 is the test substance at the selected concentration formulated in a 1:1 mixture of Freund's complete adjuvant and physiological saline. On days 6-8, a filter paper loaded with test substance in a suitable vehicle is applied to the test area covered by occlusion for 48 hours. Approximately one day before the application, if the substance is not a skin irritant, the test area is treated with 10 % sodium lauryl sulphate in vaseline, in order to create a local irritation. Control animals receive the same treatment using vehicle without test substance. Challenge is carried out on days 20-22 in treated and control animals. A patch loaded with test substance is applied to one flank of the animals, a patch with the vehicle may also be applied to the other flank. The patches are covered occlusively for 24 hours. The skin reaction is evaluated 24 and 48 hours after patch removal. A rechallenge can be done one week after the first one if necessary. According to Directive 67/548/EEC, the test substance is regarded as a sensitiser when at least 30 % of the animals show a positive response.

The GPMT is an accepted test for hazard identification of skin sensitising substances. It has been regarded as a more sensitive assay that may also, for certain substances, overestimate the sensitisation hazard for the substance tested. This is partially compensated for by the fact that a positive reaction in 30 % of the animals, compared to 15% in the Buehler test, is required for classifying the test substance as sensitiser. Shortcomings of the test are that the evaluation is not based on objective measuring parameters but on visual inspection of erythema and personal judgement. Evaluation of coloured test substances, e.g. pigments and dyestuffs, is often impossible due to staining of the skin by the test substance.

1.3.3.2 References

Magnusson and Kligman, 1969; 1970

1.3.3.3 Status of validation and/or standardisation


1.3.4 Buehler Guinea Pig Test

1.3.4.1 Short description, scientific relevance and purpose

The Buehler test uses repeated closed topical applications during induction and closed challenge. One flank is cleared of hair. A filter paper is fully loaded with test substance in a suitable vehicle. The patch is held in place by an occlusive bandage for 6 hours. On days 6-8 and 13-15, the same application is carried out. Control animals are treated similarly with vehicle only. Challenge is performed on day 27-29. The untreated flank is cleared of hair and an occlusive patch of the test substance at the maximum non-irritating concentration is applied to the posterior untreated flank of treated and control animals for 6 hours. Skin
reactions are evaluated 24 and 48 hours after patch removal. A rechallenge can be done one week after the first one if necessary. According to Directive 67/548/EEC, the test substance is regarded as a sensitis er when at least 15% of the animals show a positive response.

The Buehler test is an accepted test for hazard identification of skin sensitising substances. It has been less sensitive and may underestimate the sensitisation potential of a substance. This is partially compensated for by the fact that a positive reaction in only 15% of the animals, compared to 30% in the GMPT, is required for classifying the test substance as sensitiser. Shortcomings of the test are that the evaluation is not based on objective measuring parameters but on visual inspection of erythema and personal judgement. Evaluation of coloured test substances, e.g. pigments and dyestuffs, is often impossible due to staining of the skin by the test substance.

1.3.4.2 References
Buehler, 1965

1.3.4.3 Status of validation and/or standardisation

1.4 Available human tests

Human skin sensitisation tests have been in use the last 50 years mainly in the United States. There are a number of different human sensitisation tests available. They vary with regard to the number of induction patch tests, the placing of the patches and the use of a maximisation step. However, it is not entirely clear how useful these variations are, because validation of the tests has not kept pace with development of new tests. Also see opinion of the SCCNFP on human sensitisation tests in Section 1.1.

1.4.1 Schwartz-Peck Test
1.4.1.1 Short description, scientific relevance and purpose
Subjects receive a single topical patch application of varying dose for 24, 72 or 96 hours during the induction phase and, after a resting period of 10-14 days, are challenged with a challenge patch application for 48 hours. After patch removal, the skin reaction is scored. The Complete Schwartz-Peck test, further comprises a 4 week use test with the product after the challenge patch. The Schwartz-Peck test only detects potent sensitisers and is considered obsolete in comparison with other human sensitisation assays.

1.4.1.2 References
Schwartz and Peck (1949) and Schwartz (1951 and 1969)

1.4.1.3 Status of validation and/or standardisation
No standardised test guideline is available. The test has not undergone an official validation process.
1.4.2 Human Repeated Insult Patch Tests (HRIPT)

1.4.2.1 Short description, scientific relevance and purpose

The HRIPT is performed in at least four different forms. The concentration of material chosen for induction and challenge in the HRIPT is determined by considering the following factors: previous human experience, previous sensitisation tests in guinea pigs and irritation studies in humans. It is common practice to test multiple substances simultaneously, because it saves time and cost, but the scientific basis for multiple simultaneous inductions is not substantiated.

a) Draize test: ten consecutive induction patches are applied to new skin sites on the arms or back for 24 hours every other day 3 times a week. Each induction site is evaluated for erythema and edema after removal of the patch. Two weeks after the last induction, a challenge patch is applied for 24 hours and subsequently read. The response after challenge is compared to the responses reported after the early induction patches.

b) Shelanski-Shelanski test: it is comparable to the original Draize HRIPT but employs 15 consecutive induction patches to the same site and if erythema and/or edema develops during induction the following patch should be moved to an adjacent untreated area. 2-3 weeks after the last induction a challenge patch is applied for 48 hours and scored. The induction patch responses are also noted and interpreted as evidence of cumulative irritation.

c) Voss-Griffith test: it is also like the original Draize HRIPT with nine 24-hour patch tests conducted over a 3 weeks period and challenge is performed 2 weeks later with duplicate patches applied to the induction skin site and to the opposite arm. This assay allowed testing of four materials simultaneously. Repeated challenge is recommended in case of dubious reactions.

d) Modified Draize test: it differs from the original Draize test by subjecting the volunteers to a continuous induction period with patch exchange 3 times a week until a total of 10 patches have been applied. The patches are reapplied to the same site, and only if moderate inflammation has developed, the next patch is moved to an adjacent skin site. Challenge is performed on naive skin two weeks later with a 72-hour patch test with a non-irritating concentration of the substance.

Nowadays, the HRIPT is usually only performed to confirm the safe use of potentially sensitising substance in consumer products, such as cosmetics or household products. Other substances are not normally tested in the HRIPT. The test concentration is normally at the upper end of the suggested use concentration range and is below concentrations giving positive results in animal tests.

1.4.2.2 References

a) Draize et al., 1944, Draize, 1959
b) Shelanski and Shelanski, 1951; Shelanski, 1953
c) Voss, 1958, Griffith and Buehler, 1976
d) Marzulli and Maibach, 1973

1.4.2.3 Status of validation and/or standardisation
No standardised test guideline is available for the HRIPT. The HRIPT has not undergone a formal validation process.

1.4.3 Human Maximisation Test (HMT)

1.4.3.1 Short description, scientific relevance and purpose
The HMT includes five repeated 48-hour occlusive patch tests on the same skin site with a 24 hour rest period between removal and reapplication of the patch. Substances with irritating potential are applied in a concentration giving a moderate erythema and for substances that are non-irritating the test site is pretreated with a 24-hour patch of 5% sodium lauryl sulphate before induction. Following a two-week rest period after the last induction patch, the extent of sensitisation is evaluated by a 48-hour occluded patch test with the maximum non-irritating concentration of the substance on a slightly irritated skin site. The challenge site is scored after 24 and 48 hours after patch removal and the sensitisation index is noted. Because the human maximisation test may produce a rather dramatic effect on the skin it may be considered unacceptable today.

1.4.3.2 References
Kligman, 1966; Kligman and Epstein, 1975

1.4.3.3 Status of validation and/or standardisation
No standardised test guideline is available. The test has not undergone an official validation process.

1.5 References


SCCNFP, 2000. The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers Notes of Guidance For Testing of Cosmetic Ingredients For Their
Safety Evaluation. SCCNFP/0321/00 Final, adopted by the SCCNFP during the plenary meeting of 24 October 2000.


2. Inventory of alternative methods currently available

2.1 Alternatives to all predictive tests for skin sensitisation hazard involving chemical structure activity relationships

2.1.1 State of the art

This area of toxicological science has been under sustained investigation for the last two decades. Roberts and Williams (1982) proposed a sophisticated mathematical approach which related the physicochemical properties of a substance, and the dose at which it was applied to skin, to the degree of sensitisation which would occur. Whilst this approach led broadly to the development of quantitative structure activity relationships for several series of closely related chemicals (reviewed in Barratt, Basketter and Roberts, 1999), it also paved the way for the development of structure activity models capable of basic hazard identification. It is these which are discussed below, since traditional approaches to the establishment of QSARs seems to be likely to remain restricted for the reasonably foreseeable future to limited chemical groups. An extensive review of these topics is being published elsewhere (Cronin et al, 2003)

Relationships between the structure and biological properties of chemicals can be programmed into knowledge based expert systems. One such expert system is DEREK (“Deductive Estimation of Risk from Existing Knowledge”; Sanderson & Earnshaw, 1991; Ridings et al., 1996), which is under ongoing development by LHASA Ltd. (School of Chemistry, University of Leeds, UK). DEREK covers a variety of toxicological endpoints (e.g. mutagenicity, carcinogenicity, skin sensitisation), and is in widespread use in the chemical industry. Other expert system approaches to the prediction of skin sensitisation include the TOPKAT (TOxicity Prediction by Komputer Assisted Technology; Enslein et al., 1997) and CASE (Computer Automated Structure Evaluation) systems (Graham et al., 1996). These computer based systems are built upon varying approaches, but all employ physicochemical descriptors of chemical sensitisers and non-sensitisers as a means to provide a more general characterisation of potential allergens. TOPKAT is a suite of two modules, one for non-sensitisers v sensitisers and the other for splitting the latter group into weaker and stronger categories. Its predictions are based on physical and physical chemical properties and not on biological nor on biochemical mechanisms. Unlike DEREK it is not an expert system because it does not use rules or structural alerts. A number of different physical and physical chemical descriptors (shape indices (Kappa), symmetry indices, MW, log P, Kier-Hall electrotopological states (E-States)) are correlated with toxicity data (here skin sensitization data from guinea pig maximisation tests. Query substances are evaluated based on a structural similarity search. These computer systems are used variously by a number of agencies (see Walker et al, 2002; OECD 2002).

DEREK embodies both a controlling programme and a chemical rulebase. The chemical rulebase consists of descriptions of molecular substructures called “structural alerts”, which correlate with specific toxicological endpoints. The user communicates with DEREK by drawing the two-dimensional structure of the chemical under investigation on the screen. The rulebase is then searched against that structure, and any structural alert is highlighted, together with a message indicating the nature of the toxicological hazard.

The original skin sensitisation rulebase contained around 40 rules (Barratt et al., 1994), which were derived from an historical database (Cronin & Basketter, 1994) containing data from guinea-pig maximisation tests on 135 chemicals that had been classified as skin sensitisers
according to EU criteria, as well as a similarly sized group of non-sensitisers. As a result of
development of the system over almost 10 years (e.g. Barratt & Langowski, 1999), the
number of structural alert rules for skin sensitisation currently stands at 61 in Version 6 of the
programme. In addition, DEREK now contains a reasoning engine, which in an integrated
manner refines the output with additional information on the ability of the substance to
penetrate skin.

DEREK will serve as an example of the small number of computer based systems available
commercially which are intended to predict whether a chemical possessed the intrinsic hazard
of skin sensitisation.

2.1.2.1. DEREK

1. Short description, scientific relevance and purpose

The following two step strategy for the assessment of skin sensitisation potential is employed
when using the DEREK rulebase:

The chemical is processed through the rulebase, to see if it has the potential to react with skin
proteins either directly or, in some cases, after metabolism. If no structural alert is triggered,
either the chemical does not possess the requisite reactivity, or its reactivity is outside the
scope of the current knowledge base. In many cases, absence of chemical reactivity can be
confirmed by inspection of the chemical structure. For chemicals that do not possess the
appropriate chemical reactivity, no further computational evaluation is performed. However,
an evaluation of possible metabolic activation of the compound is considered by expert input.

For chemicals or their metabolites that do possess the appropriate chemical reactivity, the
second step is to assess their skin permeability/partition parameters. This initially involves
using either empirical or calculated values for computation of the log octanol/water partition
coefficient (logP) and/or to theoretically predict the log permeability coefficient (logKp)
(Barratt, 1995). Molecular weight and melting point values are also used in the prediction
algorithms to calculate logKp. Kp values can also be measured by using validated in vitro
skin penetration models. Using in vitro models also allows for the visualisation, as well as the
quantification, of partitioning of compounds within the skin sub-structures.

2. References – See the body of the text

3. Developer of the method – LHASA UK in collaboration with Unilever and other groups
who participate in the evaluation and evolution of the system.

4. Known users include most of the major cosmetic companies and several agencies.

5. Status of validation and/or standardisation is not straightforward to answer for a computer
system, particularly an expert rule based system, which, by definition, evolves as new
knowledge is acquired.

- Is the protocol defined/optimised? Yes, but optimisation will be
  ongoing as new knowledge enables the rules to be refined or supplemented.
- Is there data on intra-laboratory variation? Not applicable.
- Is there data on the transferability? Not applicable.
- Is there data on the inter-laboratory variability? Not applicable.
- In vivo/in vitro comparisons? These have been carried out during model development. Most recently, the BfR (Bundesinstitut für Risikoforschung - Federal Institute for Risk Assessment, http://www.bfr.bund.de/cms/detail.php?template=internet_en_index_js) examined DEREK (Zinke et al, 2002).
- Is the method validated? No.

6. What efforts are needed to complete validation of the method? Key to this is a sustained effort to refine DEREK rules with existing knowledge as well as via the acquisition of new knowledge via evaluation of chemistry areas where the system is currently weak. These are identified via simple evaluation with currently available/being prepared datasets.

2.1.2.2 Alternative computer methods, as mentioned in 2.1.1 exist, but generally they are at a similar, or often lower, status of development compared to DEREK.

2.1.3 Future prospects and recommendations

2.1.4 Key references


2.2 In vitro tests for skin sensitisation

2.2.1 State of the art

Allergic contact dermatitis (ACD) is a cell mediated immune response to small molecular weight chemicals that contact and penetrate the skin. There are a variety of characteristics that determine whether a chemical can function as a contact sensitizer (or allergen) including the ability to penetrate into the skin, react with protein and be recognized as antigenic by immune cells. The ultimate challenge for developing non-animal test methods for skin sensitization testing will be applying our mechanistic understanding of ACD to the design of predictive in vitro alternative test methods (Ryan et al, 2001). Specifically, the in vitro approach should be designed so that a chemical’s potential to penetrate the skin, react with protein/peptide (biotransformation may be required) and initiate an antigen-specific immune response is incorporated in the test methods developed. In this section, we review in vitro skin allergy research conducted on single cell cultures and the more complex models such as skin explant and skin equivalent models.

2.2.2.1 Keratinocyte cultures

1. Short description, scientific relevance and purpose

Over 90% of the cells present in the epidermis are keratinocytes (KC) and, as such, they are often the first cells in the skin to encounter chemicals which have penetrated through the stratum corneum. KC are able to produce and secrete a number of proinflammatory cytokines, chemokines and growth factors (Matsue et al., 1992), and there is evidence that they play a role in immune-mediated skin diseases including ACD (Schwarz and Luger, 1992). Wilmer et al. (1994) used a commercially available source of human KC to examine the effects of sensitizing and non-sensitizing chemical irritants on selected intracellular and secreted cytokines. The non-sensitizing irritants phenol, sodium lauryl sulfate (SLS), and croton oil induced increases in interleukin-8 (IL-8) production whereas no change or a reduction in the baseline level of IL-8 production were observed following treatment with benzalkonium chloride (BC), CrO$_3$ or the contact allergens 2,4-dinitrofluorobenzene (DNFB) or oxazolone. Croton oil, phenol, SLS, BC and DNFB were all found to stimulate the production and accumulation of intracellular interleukin-1? (IL-1?). In short, the sensitizing and non-sensitizing irritants tested could not be differentiated by the production of either IL-8 or IL-1?, and the authors concluded that a given pattern of cytokine production is chemical-specific. The effects of three contact sensitizers on IL-1? mRNA expression in human KC cultured from neonatal foreskins were found also to be varied (Pastore et al., 1995). A dose-dependent induction of IL-1? mRNA was seen following treatment with neomycin sulfate. Benzocaine produced no change in its signal, and dinitrobenzene sulfonate (DNBS) suppressed IL-1? mRNA expression. More recently, the in vitro production of IL-1? has been examined using the mouse-derived KC line, HEL30 (Corsini et al., 1998). Five allergens, two irritants and two non-sensitizers were investigated for their ability to induce extracellular (released) and intracellular (cell-associated) IL-1?. Only the contact allergens were found to increase cell-associated IL-1?, in a dose dependent fashion, while both the allergens and irritants induced the release of IL-1?. The non-sensitizing chemicals, ethanol, and glycerol, had no effect on IL-1? production. Researchers have also investigated changes in cell surface antigen expression after allergen or irritant exposure. In examining epidermal cell cultures obtained from the ears and skin of Balb/c mice, Coutant et al. (1999b) reported that the strong contact allergen trinitrobenzene sulfonic acid (TNBS) induced the expression of CD40 on KC (defined as Ia/CD45 negative cells) whereas the irritant SLS did not.
Recently, Wakem et al. (2000) demonstrated that CD80 transcriptional activity and cell surface expression in cultured human KC were up regulated to similar extents by treatment with either allergens or irritants. They hypothesized that the response of KC to chemical stimuli serves as a signal for activating T cell mediated inflammation for both ACD and irritant contact dermatitis. This suggests that the KC are simply responding to a toxic insult and are not able to distinguish allergens from irritants. Lozsekova et al. (2002) tested the effects of known allergens, namely nickel sulphate, potassium dichromate, cobalt nitrate and cytotoxic cadmium sulphate on the proteins of cellular contacts (vinculin, talin, E-cadherin, desmoplakin) and actin cytoskeleton (actin filaments) of cultivated human keratinocytes. The localisation of proteins of cellular contacts was detected by means of direct immunofluorescence. The authors reported a decrease in, and destruction of cellular contact proteins and actin cytoskeleton after testing the effect of all allergens, while the most significant changes were detected in E-cadherin, vinculin and actin filaments. Desmoplakin and talin were less damaged.

2. References - See body of the text

3. Developer of the method - Not applicable

4. Known users - None

5. Status of validation and/or standardisation - No validation of this method to date.

6. What efforts are needed to complete validation of the method? Not applicable

2.2.2.2. Langerhans cell cultures

1. Short description, scientific relevance and purpose

Langerhans cells (LC), considered to be the principle antigen presenting cell (APC) in the skin, play a key role in the development of allergic contact sensitization. LC constitute only 1-3% of all epidermal cells. Many isolation techniques have been developed to obtain purified populations of LC from human and murine sources (Hanau et al., 1988; Koch et al., 1992; Simon et al., 1995; Teunissen et al., 1988), but the numbers of cells obtained are relatively low and, to date, no LC line has been established. Therefore, the availability of sufficient numbers of these cells has been a limiting factor in the development of LC-based in vitro methods. Despite this obstacle, several investigators have focused on events which occur in LC following their exposure to chemical haptens and irritants. Changes which occur in Langerhans cell surface marker expression following in vivo chemical treatment have been explored (Aiba and Katz, 1990; Schwarzenberger and Udey, 1996). Using murine LC and flow cytometry, Herouet et al. (1999) examined the effect of chemical treatment in vitro on the expression of several LC surface markers, including MHC class I and class II, adhesion molecules CD54 and CD11c, co-stimulatory molecules CD80 and CD86, and dendritic cell-specific markers DEC-205, 4F7 and 33D1. They reported that the only surface marker affected by exposure to contact sensitizers was 33D1, a murine specific dendritic cell marker. They observed a consistent, reproducible reduction in 33D1 expression on LC within 30 minutes of exposure to strong (oxazolone, DNBS), moderate (p-phenylenediamine) and weak (mercaptobenzothiazole) sensitizers. The irritant SLS also induced a decrease in 33D1 expression. However, SLS similarly affected the other markers examined, presumably due to a surfactant effect on the integrity of the LC cytoplasmic membrane. While the exact role the 33D1 molecule plays in the immune response to contact sensitizers remains to be elucidated,
the apparently selective down-regulation of this marker by chemical haptens lends itself to further investigation in the development of \textit{in vitro} tests. Using flow cytometry, Verrier \textit{et al.} (1999) demonstrated that E-cadherin and HLA-DR expression were altered on the surface of human epidermal Langerhans cells exposed to contact sensitizers (isoeugenol, cinnamaldehyde, TNBS, Bandrowski's base, p-phenylenediamine) for 4 hours at 37°C. A dose-dependent decrease in the mean fluorescence intensity of HLA-DR was reported with no change in the number of HLA-DR positive cells. A decrease in the percentage of E-cadherin positive cells as well as a downregulation of E-cadherin expression was found following treatment with all allergens except isoeugenol. Treatment with SLS did not significantly affect either HLA-DR or E-cadherin expression. Tuschl and Kovac (2001) reported dendritic cells from peripheral mononuclear blood cells responded to NiSO$_4$, dinitrochlorobenzene, 2,4,6-trinitrobenzene sulfonic acid, alpha-hexylcinnamaldehyde and eugenol in regard to their up-regulation of the co-stimulatory molecule CD86, of intercellular adhesion molecule CD54 and of the HLA-DR antigen. The irritant sodium dodecyl sulfate (SDS) and the vehicle dimethyl sulfoxide (DMSO) had no effect.

The internalization of surface MHC class II molecules via endocytosis by antigen presenting cells is viewed as an important early step in antigen processing and is one which has been demonstrated in human (Girolomoni \textit{et al.}, 1990) and murine (Becker \textit{et al.}, 1992a,b) LC. Lempertz \textit{et al.} (1996) explored the possibility of using endocytosis of contact sensitizers by murine LC as the basis for a predictive \textit{in vitro} assay. Briefly, LC present in epidermal cell suspensions were labeled with an anti-MHC class II monoclonal antibody. By means of a flow cytometric method using second step reagents labeled with pH-sensitive fluorochromes, they found differences in the mean fluorescence intensity of the internalized label which were related to the chemical treatment of the LC. Endocytosis of the MHC complex into acidic compartments resulted in a quenching of fluorescence, whereas internalization into less acidic compartments resulted in a conservation of fluorescence intensity. Cells exposed to the solvent dimethylsulfoxide (DMSO) were used as a reference and their fluorescence intensity was defined as 100%. Treatment with irritants SLS and benzoic acid and nonsensitizing stimulatory materials concanavalin A (Con A) and phorbol 12-myristate 13-acetate (PMA) produced little to no change. Treatment with contact allergens formaldehyde, nickel sulfate, 5-chloro-2-methylisothiazolinone plus 2-methylisothiazolinone (MCI/MI), potassium dichromate, DNFB, dinitrochlorobenzene (DNCB) and trinitrochlorobenzene (TNCB) resulted in a conservation of fluorescence intensity of 1.7- to 2-fold that of DMSO treated cells. While the authors admit that the influence of hapten on receptor-mediated endocytosis is not proof of the allergenic potential of a chemical, there was a strong correlation between reactivity in their assay and \textit{in vivo} sensitizing capacity. A similar study exploring the use of MHC-II endocytosis as a means for distinguishing sensitizers from irritants was conducted by Rizova \textit{et al.} (1999) using human LC. Three different methods were used to evaluate surface expression and internalization of HLA-DR: flow cytometry, laser scanning confocal microscopy (LSCM) and electron microscopy. As observed by flow cytometry, they found that moving freshly isolated LC from 4°C to 37°C produced a decrease in the surface expression of HLA-DR which was linked to the spontaneous internalization of the MHC-II molecule. This spontaneous internalization was increased to a similar extent by pre-incubation with noncytotoxic concentrations of either sensitizers (DNFB, diphenycyprone, oxazolone and 3-n-pentadecylcatechol) or irritants (SLS, BC and benzoic acid). Thus, no clear differences between the two classes of chemicals were detectable by flow cytometry. However, when observed by LSCM, cells which had been treated with the sensitizers internalized the HLA-DR molecules into large vesicles which exhibited a very bright fluorescence in contrast to irritant treated cells which were similar to untreated controls, with HLA-DR molecules in small vesicles showing a diffuse fluorescence. Clear differences were
also observed using electron microscopy. HLA-DR molecules were found preferentially in lysosomes collected near the nucleus in sensitizer treated LC, while the MHC-II molecules were observed at the cell membrane surface and in prelysosomes in irritant treated and control LC.

The induction of tyrosine phosphorylation following stimulation with contact sensitizers has been examined in both human (Kühn et al., 1998) and murine (Neisius et al., 1999) LC. Whereas freshly isolated human LC failed to demonstrate changes in phosphotyrosine (p-tyr) following exposure to the strong hapten MCI/MI, 24 hour cultured LC demonstrated a significant increase in p-tyr by flow cytometric quantitation (Kühn et al., 1998). Neisius et al. (1999) reported similar increases in p-tyr in murine LC following in vitro stimulation with the strong contact sensitizers TNCB and MCI/MI but not with the irritants SLS or benzoic acid. Although poorly defined, the mechanisms of signaling pathways in LC during hapten-mediated activation may serve as a basis for the development of an in vitro test system.

Following an encounter with antigen, LC have been shown to migrate out of the skin to regional draining lymph nodes (Kripke et al., 1990; Macatonia et al., 1987). In an attempt to reproduce this event in an in vitro system, Kobayashi et al. (1994) examined the influence of hapten application on LC migration through a reconstituted basement membrane matrix. LC-enriched cell suspensions obtained from human skin were treated in vitro with either hapten (trinitrobenzenesulfonic acid (TNBS)), or fluorescein isothiocyanate (FITC)), or irritant (SLS). The treated cells were then seeded into the upper compartment of a modified Boyden-chamber over a Matrigel-coated polycarbonate filter underlayed with a cellulose nitrate filter, with fibroblast conditioned medium in the lower compartment to act as a chemoattractant. The number of HLA-DR positive cells on the upper surface of the cellulose nitrate membrane were counted to quantify LC migration. Hapten treated LC were found to exhibit a time-dependent increase in migration into the lower filter compared to non-treated cells while the migratory pattern of SLS treated cells did not differ. While donor to donor variability in the absolute extent of LC migration was noted, hapten treatment enhanced the migration compared to untreated cells in every suspension studied. The simplicity of this method lends itself to further investigation using a chemical set with a wider range of allergenic potencies.

2. References - See body of the text

3. Developer of the method - Not applicable

4. Known users - None

5. Status of validation and/or standardisation - No validation of test methods to date.

6. What efforts are needed to complete validation of the method? Not applicable.

2.2.2.3 Peripheral blood derived dendritic cells

1. Short description, scientific relevance and purpose

Dendritic cells (DC) are a distinct group of leukocytes characterized by their unique morphology and their ability to initiate immune responses by processing and presenting antigens. As mentioned previously, the ability to obtain large numbers of LC from skin is a limiting factor for their use in the development of in vitro methods to assess contact sensitization potential. Therefore, the development of culture techniques to generate DC from
CD34+ precursors, either from bone marrow or cord blood, and peripheral blood mononuclear cells has provided a source of LC-like antigen presenting cells for study. In addition to having typical DC morphology, peripheral blood mononuclear cell (PBMC)-derived DC express surface markers consistent with bone marrow derived DC, and are capable of eliciting a primary allogeneic mixed lymphocyte response (Romani et al., 1994). While minor variations in culture techniques and cytokine induction cocktails have been reported, to date there is no one method for generating DC from PBMC that investigators agree upon as standard.

In an attempt to develop an in vitro model for contact sensitization, Degwert et al. (1997) examined phenotypical alterations of human blood-derived DC under the influence of subtoxic concentrations of different chemicals and contact sensitizers. They showed that incubation with contact sensitizers urushiol, primin, C10- and C11-primin analogues, alantolactone, isoalantolactone and NiSO₄ resulted in a decrease of HLA-DR expression on the surface of the DC, if the incubation period did not exceed 3 hours. Incubation with irritants like SLS and BC did not change DR expression under the same conditions. Adhesion molecule expression (ICAM-1 (CD54)) showed no differences between irritants and contact sensitizers. The authors summarized by saying that this system can be used to discriminate between contact sensitizers and irritants. Using longer incubation times (24 and 48 hr), Aiba et al. (1997) reported that haptens such as NiCl₂ and DNCB cause a significant increase in the surface expression of CD54, CD86 and HLA-DR on monocyte derived DC compared to non-treated controls or to DC treated with irritants (ZnCl₂, SDS, or BC). However, they noted that the changes in expression of these markers were quite variable among the subjects tested, with some subjects having no change at all. With similar findings, Coutant et al. (1999a) illustrated that monocyte derived DC expressed higher surface marker expression of HLA-DR, CD86, CD40 and CD54 in the presence (48 hrs) of haptens (aminophenol, chlorpromazine hydrochloride, DNCB, nickel sulfate) as compared to irritants (SDS, benzoic acid). After incubation with haptens, but not with irritants, they observed a higher production of TNF-a. In their experience, no release of IL-1β, IL-10 or IL-12 was detected. Compared to the activation elicited by haptens, stimulation with staphylococcal enterotoxin B (SEB) induced strongly upregulated HLA-DR and co-stimulatory molecule expression. They concluded that the data underlines the activating potential of haptens versus irritants on DC functions and that the differing levels of DC activation suggest the involvement of distinct cellular events. Using Langerhans cell-like DC generated from CD34+ cord blood cells cultured for 8 days with GM-CSF and TNF-α, Rougier et al. (2000) demonstrated that incubation with hapten for 48 hrs induced DC maturation, based on phenotypic changes such as the expression of CD83, increased expression of HLA-DR and CD86, and a decrease in E-cadherin. All of these changes were observed following treatment with the strong allergen BB, and not with the irritant SDS. However, the weaker allergens tested, PPD, citronellal and coumarin, mostly induced changes in CD86 expression and not HLA-DR or CD83 which, were observed only in a limited number of subjects (1 or 2 out of 8). Hulette et al. (2002) examined expression of HLA-DR, CD54, CD80, CD86 on DC treated 48 hours with dinitrofluorobenzene (DNFB) and methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), the irritant sodium dodecyl sulfate (SDS), lipopolysaccharide (LPS), and tumor necrosis factor alpha (TNF-α). Treatment of PBMC-DC with either MCI/MI or DNFB induced a slight upregulation of class II major histocompatibility (MHC) expression (HLA-DR), whereas LPS and TNF-α significantly upregulated CD54 and slightly upregulated CD80 and HLA-DR expression. For KG-1 DC (Hulette et al, 2001), only MCI/MI upregulated CD86 expression, whereas TNF-α upregulated CD54 and slightly upregulated CD80 and CD86 expression. SDS had no effect on surface marker expression in either PBMC-DC or KG-1 DC. Changes in surface marker expression in PBMC-DC treated with chemical
allergens were detected in 2 of 5 donors, suggesting a limited sensitivity of PBMC-DC under these defined isolation and culture conditions. The conclusion was that under these culture and treatment conditions, measurement of surface marker changes in vitro using PBMC-DC or KG-1 DC does not provide a sensitive in vitro method with sufficient dynamic range for assessing the contact sensitization potential of a chemical. Recently, Banerjee et al. (2003) have proposed measurement of IL-1α and IL-1β protein levels to distinguish allergens from irritants. They report that the allergens induced the secretion of IL-1α at concentrations two- to five-fold higher than those of controls, depending on the concentration and the particular irritant.

As discussed earlier, the internalization of surface MHC class II molecules via endocytosis by antigen presenting cells is viewed as an important early step in antigen processing and is one which has been demonstrated in human (Girolomoni et al., 1990) and murine (Becker et al., 1992a,b) LC. As a surrogate to LC, Becker et al. (1997) used peripheral blood-derived DC with a flow cytometric method to screen for the modulation of receptor-mediated endocytosis. Briefly, a method was established to monitor the influence of chemicals in the intracellular targeting of antibody-crosslinked MHC class II molecules after their uptake by human DC. The assay is based on the pH-sensitivity of internalized fluorescein-coupled MHC class II specific antibodies. Untreated DC showed quenching of fluorescence intensity due to internalization into acidic intracellular compartments, whereas fluorescence intensity was conserved in DC treated with strong contact sensitizers (DNFB, MCI/MI, imidazol urea). The authors summarize by stating that the data suggest that the capacity of a chemical to modulate endocytotic mechanisms in DC in vitro reflects the probability of that substance acting as a hapten in vivo.

IL-1α is the first cytokine which is rapidly produced by LC after topical application of contact sensitizers onto mouse skin (Enk and Katz, 1992). This early up-regulation of IL-1α was specific for contact sensitizers and not irritants. These data strongly suggest that IL-1α plays an essential role for the induction of primary immune reactions like allergic contact dermatitis in the skin (Enk et al., 1993). Therefore, IL-1α expression may provide an alternative approach to the assessment of the sensitizing activity of chemicals. Reutter et al. (1997) investigated the ability of five contact sensitizers and one irritant to induce IL-1α gene expression in vitro in PBMC-DC. DC were cultured in serum free medium supplemented with GM-CSF and IL-4 for 5 days. Then the DC were pulsed for 30 minutes at 37°C with subtoxic concentrations of the contact sensitizers pentadecyl-catechol, TNBS, DNFB, NiSO₄, K₂Cr₂O₇ and the irritant SDS. Total RNA was extracted from the DC and IL-1α mRNA expression was detected using reverse transcriptase-polymerase chain reaction (RT-PCR). They reported that all contact sensitizers increased IL-1α gene expression, whereas treatment with the irritant SDS had no significant effect. They concluded that they had developed an in vitro system which may be useful to evaluate the allergic potentials of chemicals and products. De Smedt et al. (2001) reported modulation of phenotype and cytokine production in CD34+ derived DC treated with NiCl₂ and SDS. In a later paper (De Smedt et al, 2002), the authors reported that monocyte-derived dendritic cells were more robust in response to allergens then CD34+ progenitor cells. Pichowski et al. (2000) confirmed that potent contact sensitizers selectively up-regulate IL-1α mRNA expression, but observed this increase from a limited number of donors (4 out of 9) after exposure in vitro to the potent sensitizer DNFB. The variation in DNFB-induced upregulation of IL-1α expression was shown to be donor dependent and not due to inter-PCR variation. They also confirmed that the irritant SLS did not increase IL-1α mRNA levels, even in DC derived from individuals sensitized to DNFB. In a later paper, Pichowski et al. (2001) reported that only modest responses were being
observed with very strong allergens and that differences in responder/non-responder phenotypes were being detected. They concluded that the measurement of IL-ß in human blood-derived DCs was not likely to lend itself to a routine assessment of skin sensitizing activity.

Recently it was shown that the use of DC derived from pooled monocytes from different donors reduced the inter-individual and inter-experimental variations of the cell surface expression of CD 86, the upregulation of IL-1? mRNA expression and the downregulation of aquaporin P3 mRNA expression. These parameters showed marked changes in response to TNBS and two aromatic amines used as hair dyes (p-toluylenediamine (PTD) and hydroxyethyl-p-phenylenediamine (HE-PPD)), but no alteration in response to SLS. Both aromatic caused marked DC activation, comparable to the effects of the known skin sensitisier TNBS. The seemingly contradictory results in the LLNA, in which PTD was identified as a skin sensitisier whereas HE-PPD was not sensitising, were only resolved when the skin penetration capacity, which was shown to be about 200-fold lower for HE-PPD compared to PTD, was taken into account (P. Aeby, C. Wyss, H. Beck, P. Griem, H. Scheffler, and Carsten Goebel, J. Invest. Dermatol., accepted for publication).

In addition to changes in cell surface marker expression, Aiba et al. (1997) also examined the production of cytokines by monocyte-derived DC. They found that cytokine production was dependent on the chemical treatment. DC exposed to NiCl2 had a significant increase in the production of IL-1?, IL-6 and TNF-? protein, while DNCB treatment only induced an increased secretion of IL-1?. Lore et al. (1998) developed a direct immunocytochemical method to identify cytokine and chemokine production in peripheral blood-derived DC at the single cell level. This method was used to assess TNF-a, IL-1a, IL-1ra, IL-6, IL-8, IL-10, IL-12, GM-CSF, MIP-1a, MIP-1ß, and RANTES. IL-1ra and IL-1a were expressed in 10-25% of unstimulated cultured DC, while all the other cytokines tested were undetectable. IL-1ra, IL-1a, and IL-1ß were expressed in 85% of DC after 3 hr of lipopolysaccharide stimulation. The investigators did not explore DC which had been treated with haptens, but this approach might find utility as a predictor of DC activation. Aiba et al. (1999) showed the activation and apoptosis of peripheral blood derived DC when treated for 24 hrs in culture with various contact sensitizing agents using Annexin V and TUNEL methods. Aiba et al. speculate that it is conceivable that chemicals, more specifically the contact sensitizers, induce a stress response that activates DC. However, because NiCl2 and DNCB induce DC activation by different mechanisms and because different chemicals induce heterogeneous responses in the augmentation of costimulatory molecules or class II MHC antigen, cytokine secretion and induction of apoptosis, the activation of DC cannot be explained simply as a result of a stress response. Aiba et al. postulate that these chemicals may directly affect the intracellular signal transduction of DC. Although not viewed as an alternative method to predict the skin sensitization potential of chemicals, apoptosis is emerging as a parameter for exploration in hapten/DC interactions.

2. References - See body of the text

3. Developer of the method - Not applicable

4. Known users - None

5. Status of validation and/or standardisation - No validation of test methods to date

6. What efforts are needed to complete validation of the method? Not applicable
2.2.2.4 Cell lines
In addition to using LC or DC, which are difficult to isolate and to get sufficient numbers, investigators have been evaluating various cell lines to use as dendritic cell surrogates. For example, an LC-like murine cell line, XS52, has been developed from the epidermis of newborn BALB/c mice which displays many of the morphologic, phenotypic and functional characteristics of freshly isolated LC (Xu et al., 1995a). XS52 cells are similar to LC in terms of cytokine and cytokine receptor mRNA profiles (Ariizumi et al., 1995; Takashima et al., 1995; Xu et al., 1995b) and they have been shown to up-regulate IL-1? mRNA and secrete relatively large amounts of IL-1? protein upon antigen-dependent interaction with T-cells (Kitajima et al., 1995). In addition, it has been shown recently that mature DC can be generated from these cells using soluble factors (Yamada and Katz, 1999). While not available commercially, the XS52 cell line, with its similarities to LC, holds promise for the development of a wholly in vitro method for sensitization testing.

Using the THP-1 cell line, Yoshida et al. (2003) have reported recently that one gets augmentation of CD54 and CD86 expression in THP-1 cells treated with allergens. The investigators used flow cytometry and the mean fluorescence intensity to show the changes in the THP-1 cell line. Similarly, Ashikaga et al. (2002) reported an upregulation of CD86 and class II MHC internalization on THP-1 cells treated for 24 hours with DNCB. They stated that the upregulation was enhanced when using gamma interferon along with their allergen treatment.

2. References - See body of the text
3. Developer of the method - Not applicable
4. Known users - None
5. Status of validation and/or standardisation - No validation of test methods to date
6. What efforts are needed to complete validation of the method? Not applicable

2.2.2.5 Co-culture systems
Since the induction of contact allergy involves the interaction between LC and T-cells, a culture system which contains both stimulating APC and responding T-cells, would appear to provide the best approach for the development of an in vitro method for predictive sensitization testing. Using hapten treated cultured murine LC, Hauser and Katz (1988) were able to demonstrate primary activation and proliferation of naïve T-cells in vitro. LC modified with the chemical allergens TNBS or FITC elicited a strong proliferative response in non-sensitized T helper cells. In addition, these in vitro sensitized T-cells proliferated in response to re-stimulation in vitro with hapten-modified spleen cells. A similar method was used by Moulon et al. (1993) to examine the ability of human LC to sensitize autologous T-cells. They found that 2 day cultured LC, but not freshly isolated LC, treated with TNBS were capable of inducing primary T cell proliferative responses in vitro. The cultured LC were shown to be very potent APC, with T-cell responses observed at a responder to stimulator ratio (R:S) as high as 100:1. The hapten-specificity of the primary T cell response was confirmed by the capacity of the T cells to proliferate in secondary response upon re-
stimulation with TNBS treated LC. Krasteva et al. (1996) utilized this methodology in an attempt to develop a predictive in vitro screening assay for contact sensitizers. T-cell proliferation, as determined by \( ^3 \text{H} \)-thymidine incorporation, resulting from stimulation with hapten-modified LC was assessed for strong (TNBS, FITC), moderate (\( \mu \)-phenylenediamine (PPDA) and its oxidation product Bandrowski’s base (BB)), and weaker (citronellal, hydroxycitronellal, coumarin) sensitizers as well as the irritant SLS. Only the strong allergens (TNBS, FITC, BB) consistently induced a proliferative response.

A co-culture system of Langerhans cells integrated into human reconstructed epidermis has been developed by Régnier et al. (1997). The authors have recently reported Langerhans cells morphological changes and up regulation of the surface marker CD86 following exposure to the known sensitiser DNFB and NiSO\(_4\), and to UV-irradiation. The irritant SLS did not induced the same effects (Facy et al., 2004).

While LC are the primary APC in the skin, obtaining sufficient numbers for use is often an issue so the ability of alternative antigen presenting cells to stimulate T cells in culture has been explored. Human peripheral blood monocytes, cultured with GM-CSF alone, or GM-CSF and IL-4, have been shown to be capable of inducing in vitro primary sensitization of T cells to trinitrophenyl (TNP; the actual hapten resulting from TNBS treatment of APC), whereas freshly isolated monocytes stimulated either a weak or no response (Kobel et al., 1996). In these studies, the R:S averaged 5:1. Similar attempts to induce primary sensitization to the hapten diphenylcyclopropenone failed. Adherent peripheral blood mononuclear cells cultured in the presence of GM-CSF for 24 hours, that had been derivatized with the hapten DNCB, were shown to be capable of stimulating both proliferation and IFN-\(?\) production in naïve autologous T cells in vitro (Dai and Streilein, 1998). However, the efficiency of these APC was rather low, as the R:S used for inducing the T cells was 1:1. Using DC derived from CD34+ cord blood cells as APC, Rougier et al. (1998) reported findings similar to those of Krasteva et al. (1996): strong allergens such as TNBS, FITC and BB induced significant proliferation in naïve autologous lymphocytes, whereas the irritant SLS, and the less potent allergens (PPDA, citronellal and coumarin) tested, failed to induce a significant T cell response. Overall, these APC were fairly efficient at presenting hapten to naïve T cells as they were capable of inducing proliferative responses at R:S of 100:1. Recently, using a modification of their original procedures, Rougier et al. (2000) have been able to produce primary T cell proliferative responses to weaker allergens, PPDA, citronellal and coumarin, in a limited number of subjects. Langerhans-like dendritic cells (LLDC) were generated from CD34+ cord blood progenitors by culture for 8 days in the presence of GM-CSF and TNF-\(?\). The LLDC were then incubated for an additional 48 hrs with non-cytotoxic concentrations of the hapten which, presumably, induced maturation of the LLDC and thus increased their immuno-stimulatory capacity. The authors suggest that hapten induced phenotypic and functional maturation of the LLDC may be used as a screening test for strong allergens but detection of weaker allergens would require complementary studies.

Rustemeyer et al. (1999) have reported on a method for the in vitro priming of naïve T cells using hapten treated autologous DC derived from peripheral blood. Hapten-specific T cells to nickel sulphate and 2-hydroxyethyl-methacrylate (HEMA) were generated by their culture system in which DC, matured by exposure to TNF-\(?\) following hapten treatment, were cultured with autologous T cells for 7 days in the presence of a cytokine cocktail consisting of IL1-\(?\), IL-2 and IL-7. The hapten-specificity of the primed T cells was demonstrated by secondary proliferative responses. While the authors did not propose the culture system as a method for predictive sensitization, their protocol does appear to provide a means of priming naïve T-cells in vitro.
An elaborate co-culture system which used hapten-conjugated Pam 212 cells (a murine keratinocyte cell line) to induce a primary sensitization response in T cells from non-sensitized mice was developed by Yozeki et al. (1995). Briefly, mono-layer cultures of Pam 212 cells were incubated with test chemicals, washed, then fixed with 3% paraformaldehyde. T cells from non-sensitized mice which had been depleted of autoreactive cells, along with spleen derived macrophages, were cultured with the chemically-modified Pam 212 cells for 5 days. The T cells were then harvested and re-stimulated with mitomycin c treated, hapten-conjugated spleen cells at an R:S of 5:1 for 3 days. A stimulation index (SI) was calculated by dividing the dpm obtained in the presence of hapten modified spleen cells by that obtained in the presence of unmodified spleen cells. A number of chemicals were tested in this culture system including four strong sensitizers (oxazolone, TNP, dinitrophenyl, and FITC), three potent sensitizers (PPDA, nickel chloride and potassium dichromate), two corticosteroids (betamethasone and budesonide) and one irritant (methyl salicylate). The SIs produced by the strong sensitizers were approximately 4.0 while the potent sensitizers SIs were around 2.0-2.5. The SIs for the corticosteroids and the irritant were less than 2.0. A further examination of this method and a comparison with the guinea pig maximization test with 11 allergens and two irritants was conducted (Arimura et al., 1998). Three of the five chemicals classified as moderate sensitizers and all of the strong sensitizers were positive in both the in vitro test and the guinea pig maximization test. While the performance of this test method warrants further investigation, it is not a non-animal alternative as it relies on mice as a source of cells, though the numbers required are rather few (4 mice to determine the allergenic potential of 13 chemicals).

2. References - See body of the text

3. Developer of the method - Not applicable

4. Known users - None

5. Status of validation and/or standardisation - No validation of test methods to date

6. What efforts are needed to complete validation of the method? Not applicable

2.2.2.6 Human skin equivalent / reconstituted epidermis cultures

In vitro three-dimensional cultures of living human skin generally fall into two categories: epidermal or skin equivalents. Epidermal equivalents consist of keratinocytes which, when cultured on a filter or matrix at the air-liquid interface, develop into a fully differentiated epidermis with a stratum corneum. Cell viability, histological changes and the release of IL-1? and prostaglandin E\(_2\) (PGE\(_2\)) by the EpiDerm? model following treatment with irritants and allergens were examined by Kubilus et al. (1996). They found that the dose response curves for the release of IL-1? and PGE\(_2\) following treatment with contact irritants reflected the cytotoxicity of the dose (i.e. the higher the cytokine release, the greater the cytotoxicity). However, following treatment with contact allergens, the amount of cytokine released did not correspond directly to the degree of cytotoxicity as more IL-1? and PGE\(_2\) were produced at non-cytotoxic doses of the chemical and varied with the allergen. The SkinEthic model was used by Coquette et al. (1999) to measure IL-1? and IL-8 mRNA and protein levels as well as cytotoxicity following irritant and allergen treatment. More recently, Coquette et al. (2003) have suggested that determination of IL-8, with IL-1?, and MTT conversion shows potential to discriminate and classify irritants and allergens in a single assay. Like Kubilus et al., they report that IL-1? release increases with cytotoxicity after treatment with the irritants Triton
X100 and BC, but had no effect on IL-8 levels. In contrast, the allergen DNCB did not induce IL-1? but instead, elicited elevated IL-8 levels. However, when mRNA expression was measured, BC, Triton X100 and DNCB all induced an up-regulation in message for IL-8 while only BC up-regulated IL-1? mRNA.

The results of a number of studies conducted to assess the ability of irritants and contact allergens to affect cytokine mRNA levels in skin equivalents have been reviewed by Gerberick and Sikorski (1998). Data from those studies indicated that while cytokine message could be modulated by chemical treatment, each chemical appeared to produce changes in cytokine mRNA expression which was unique to the chemical and was also concentration and or time-dependent. No single cytokine or profile of cytokines were identified which were predictive for sensitization potential. However, Corsini et al. (1999) recently reported that interleukin-12 (IL-12) is selectively induced in the Episkin? model by treatment with chemical allergens. Following a 3 hour exposure to test chemicals, mRNA levels for both IL-12 p40 and IL-12 p35 were found to be up-regulated by contact allergens (oxazolone, eugenol and DNCB) but not the irritants (SLS and BC).

2. References - See body of the text

3. Developer of the method - Not applicable

4. Known users - None

5. Status of validation and/or standardisation – No validation of test methods to date.

6. What efforts are needed to complete validation of the method? Not applicable

2.2.2.7 Human skin explant cultures

Skin explant cultures utilize full thickness human skin specimens typically obtained from breast or abdominal reduction surgery. Like skin equivalent cultures, explants allow for the topical application of test chemicals. However, in addition to keratinocytes and fibroblasts, explants also contain cells of the immune system including LC, as well as other resident skin cells such as melanocytes and endothelial cells. A predictive assay for contact allergens which utilizes human skin explant cultures has been proposed by Pistoor et al. (1996). The assay focuses on changes in the distribution of the LC population following the epicutaneous application of test chemicals at nontoxic concentrations. They observed, in cryostat sections of the explants after 24 hours of culture, that contact allergens, but not irritants or inactive chemicals, induced a dose dependent reduction in the number of LC (HLA-DR’CD1a+ cells) in the epidermis and an accumulation of the remaining LC at the epidermal-dermal junction. However, LC migration in skin explants may not be a reliable endpoint for an in vitro assay for contact allergens, as LC have been shown to spontaneously migrate out of human skin cultured for one to three days (Lukas et al. 1996). Further studies by the same laboratory examined changes in cell surface molecules and cytokine expression by LC in the skin explants following chemical treatment (Rambukkana et al. 1996). They reported that after 24 hours of exposure to contact allergens, the LC which had migrated to the epidermal-dermal junction had a decreased expression of both CD1a and HLA-DR and a significant increase in the expression of ICAM-1 (CD54). The presence of IL-1? was detected in epidermal cells as early as 4 hours following treatment with the contact allergen DNFB, but not with the irritant SDS or the vehicle. After 24 hours of DNFB treatment, IL-1? as well as TNF-?, IL-1?, GM-
CSF and IL-6 were evident in the epidermal cells. However, these cytokines were also found in the explants treated with SDS.

2. References - See body of the text

3. Developer of the method – Not applicable

4. Known users - None

5. Status of validation and/or standardisation – No validation of test methods to date.

6. What efforts are needed to complete validation of the method? Not applicable

2.2.3 Future prospects and recommendations

The two - not mutually exclusive - approaches for the substitution of in-vivo sensitisation tests are in-silico methods, i.e., computer-based expert or QSAR systems, and in-vitro methods that a mostly based on chemical-induced responses of cell-culture systems. The fundamental difference between the two approaches is that more experience has been gained to date with regard to the validation of tests and the development of test guidelines (such as the OECD technical guidelines) for in-vitro alternatives.

However, at the moment in-vitro systems for the identification of skin sensitisers are still at basic research level. The advances in basic immunological research continues to put more and more candidates on the list of potential test parameters, i.e. the upregulation or downregulation of the expression of cell membrane proteins and cell-cell signalling molecules, such as interleukins, and changes in the antigen uptake process. Here, a great deal of focused research will be necessary to identify the most relevant parameters and to standardise testing protocols and validate tests. As depicted in the table, every in-vitro test will only be able to assess, at best, one step of the multi-step mechanism of skin sensitisation. This, in turn, means that no single in-vitro test parameter alone will show a sufficient correlation with the sensitising potential in vivo. The validation process has to take this into account: rather than separate validation of one or a few tests a joint validation of an in-vitro test battery is likely to be required.

Some more fundamental questions will have to be answered for in-silico systems: How should QSAR systems be validated? How can testing guidelines be developed for QSAR systems? Would such a guideline have to define the data set that is to be used with a certain QSAR system and the prediction space, i.e. prerequisite in terms of certain physico-chemical parameters that a test substance has to fulfil in order to generate meaningful QSAR results. In view of the fact that QSARs currently seem further developed than in-vitro alternatives, it is recommended that discussion of these questions among all stakeholders be initiated and further development supported.

The ability of QSAR systems and in-vitro batteries to correctly predict a chemical's property as a human sensitiser or a human non-sensitiser will constitute the decisive measure in identifying the most relevant alternative to animal testing.
2.2.4 Key references


### Summary of the alternative methods currently available and foreseeable time to achieve ESAC endorsement

<table>
<thead>
<tr>
<th>Current endpoints addressed in animal test</th>
<th>Alternative tests available</th>
<th>Endpoints measured</th>
<th>Purpose</th>
<th>Area(s) of application</th>
<th>Validation status</th>
<th>Regulatory acceptance</th>
<th>Comments</th>
<th>Estimated time to have the method validated (ESAC endorsement)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLNA&lt;br&gt;(OECD TG 429)&lt;br&gt;Lymphocytes proliferation</td>
<td>In silico alternatives (Q)SARs</td>
<td>Partial replacement&lt;br&gt;(test battery)</td>
<td>All chemicals</td>
<td>Optimised</td>
<td>None but limited acceptance by some national authorities</td>
<td></td>
<td></td>
<td>5 - 6 years</td>
</tr>
<tr>
<td>MEST&lt;br&gt;Ear swelling</td>
<td>Protein / peptide binding</td>
<td>Protein / peptide binding</td>
<td>All chemicals</td>
<td>R&amp;D</td>
<td>---</td>
<td></td>
<td></td>
<td>5 - 6 years</td>
</tr>
<tr>
<td>GPMT&lt;br&gt;Buehler Test&lt;br&gt;(OECD TG 406&lt;br&gt;EU B.06)&lt;br&gt; Skin reaction</td>
<td>Keratinocyte cultures&lt;br&gt;Keratinocyte activation (e.g. production of proinflammatory cytokines)</td>
<td>Partial replacement&lt;br&gt;(test battery)</td>
<td>All chemicals</td>
<td>R&amp;D</td>
<td>---</td>
<td></td>
<td></td>
<td>5 - 6 years</td>
</tr>
<tr>
<td></td>
<td>Langerhans cell cultures&lt;br&gt;Activation of Langerhans cells and dendritic cells (e.g. migration, cell surface marker expression, cytokine production)</td>
<td>Partial replacement&lt;br&gt;(test battery)</td>
<td>All chemicals</td>
<td>R&amp;D</td>
<td>---</td>
<td>These approaches will be evaluated in parallel with only the most successful emerging for formal validation.</td>
<td></td>
<td>5 - 6 years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Co-culture systems</strong></th>
<th>Activation of T lymphocytes (e.g. proliferation, cell surface markers expression)</th>
<th>All chemicals</th>
<th>R&amp;D</th>
<th>---</th>
<th>10 - 12 years</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reconstituted epidermal cultures</strong></td>
<td>Activation of keratinocytes (and skin penetration)</td>
<td>All chemicals</td>
<td>R&amp;D</td>
<td>---</td>
<td>10 - 12 years</td>
</tr>
<tr>
<td><strong>Human skin explant cultures</strong></td>
<td>Skin penetration and activation of keratinocytes and Langerhans cells</td>
<td>All chemicals</td>
<td>R&amp;D</td>
<td>---</td>
<td>Never, due to insufficient supply of test material</td>
</tr>
<tr>
<td><strong>In vitro Test Battery</strong></td>
<td>Various (see above)</td>
<td>All chemicals</td>
<td>R&amp;D</td>
<td>---</td>
<td>Guidance for the validation of test batteries has to be developed</td>
</tr>
</tbody>
</table>

* This table estimates the time needed to achieve ESAC endorsement for individual alternative tests assuming optimal conditions. It does not indicate the time needed to achieve full replacement of the animal test, nor does it include the time needed to achieve regulatory acceptance. “Optimal conditions” means that all necessary resources, for example technical, human, financial and coordination, are met at all times in the process and that the studies undertaken have successful outcomes.
Conclusions

Different *in silico* and *in vitro* models are being developed to assess the skin sensitising potential of chemicals and products. The two - not mutually exclusive - approaches for the substitution of *in-vivo* sensitisation comprise computer-based expert or QSAR systems, and *in-vitro* methods that are mostly based on chemical-induced responses of cell-culture systems.

Among these, (Q)SARs systems are the most developed and it is envisaged that further optimisation of such methods, achievable by extending their range of chemical knowledge, will lead to the formal validation within the next few years.

The development process of (Q)SARs is mainly carried out by consortia and companies that develop and distribute these programs. An approach to the evaluation of these systems is being defined at OECD level. In November 2002, the 34th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology held a Special session on QSARs, during which it identified a lack of validated QSARs for use in regulatory assessment and agreed to start an activity aimed at the development of internationally accepted validation principles and procedures, and evaluation of existing and promising QSARs against these principles. Six principles for judging the validity of (Q)SARs were agreed at the International Council of Chemical Associations (ICCA)/European Chemistry Industry Council (CEFIC) Workshop on “Regulatory acceptance of QSARs for Human Health and Environmental Endpoints” held in Setubal, Portugal on 4-6 March 2002. These were further discussed at the first meeting of the OECD Ad Hoc Expert group on QSARs on 31 March- 2 April 2003. The Expert group developed a draft 2 year work plan (endorsed at the 35th Joint Meeting) which comprises three activities: 1) evaluating the so-called “Setubal” principles for QSARs; 2) developing guidance documents (e.g. on the validation of QSARs); and 3) making validated QSARs available to users. Via the work of ECVAM and the ECB, the European Commission is playing a leading role in the OECD QSAR activity. The validation of (Q)SARs for skin sensitisation is a high priority for ECVAM.

Although cell based systems, have in some cases been shown to be capable of distinguishing between sensitisers and non-sensitisers (and so could be used for priority setting), they are still at a basic research level. Advances in basic immunological research continue to increase the number of potential test parameters, eg. the upregulation or downregulation of the expression of cell membrane proteins and cell-cell signalling molecules, such as interleukins, and changes in the antigen uptake process. Further research and refinement activities are needed before a test battery can be set up from the *in vitro* tests. Research is currently carried out by individual companies and academia, with funding mainly provided by industry and industry associations (i.e. a COLIPA research program). A part of the currently ongoing basic research focuses on the elucidation of the mechanisms underlying skin sensitisation. Therefore, a coordinating advisory body could help to focus research on the development of a predictive test method and, thus, speed up the R&D process.

Due to the complexity of the mechanisms of skin sensitisation, a single test will not be able to replace the currently required animal experiments. Efforts are still needed to identify the most relevant endpoints and in the optimisation of existing tests. However, combination of several *in vitro* tests, covering all relevant mechanistic steps of skin sensitisation, into a test battery can likely lead to replacement of the *in vivo* tests. The validation process has to take this into account: rather than separate validation of one or a few tests a joint validation of an in-vitro test battery will be required. Currently no guidance for the validation of a test battery is
available and development of such guidance would help the development of an animal alternative. It is proposed that such guidance be developed by ECVAM.

An indicative time foreseen for the availability of ESAC endorsed validated alternative test battery for skin sensitisation is of ten-twelve years; twelve-fourteen year is the time required for regulatory acceptance at the EU level. This estimated deadline for phasing out the animal experiments can only be met if the necessary resources are made available to progress research, development, and optimisation in this field.