Comment

An Integrated Decision-tree Testing Strategy for Skin Sensitisation with Respect to the Requirements of the EU REACH Legislation

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Summary — This report presents some of the results of a joint research project, sponsored by Defra and conducted by FRAME and Liverpool John Moores University, on the status of alternatives to animal testing with regard to the European Union REACH (Registration, Evaluation and Authorisation of Chemicals) system for the safety testing and risk assessment of chemicals. The project covered all the main toxicity endpoints associated with the REACH system. This report focuses on the use of alternative (non-animal) methods (both *in vitro* and *in silico*) for skin sensitisation testing. The manuscript reviews *in vitro* tests based on protein-ligand binding, dendritic/Langerhans cells and T-lymphocyte activation, and also the QSAR models and expert systems available for this endpoint. These tests are then incorporated into an integrated, decision-tree testing strategy, which also includes the Local Lymph Node Assay (in its original and new reduced protocols) and the traditional guinea-pig tests (which should only be used as a last resort). The aim of the strategy is to minimise the use of animals in testing for skin sensitisation, while satisfying the scientific and logistical demands of the EU REACH legislation.

Key words: *animal testing, decision-trees, Defra,* in silico *models,* in vitro *testing, integrated testing strategies, REACH, risk assessment, skin sensitisation, Three Rs.*

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Project Background

This report is one of a series of publications which present the findings and recommendations of a Defra-funded research project on the availability of alternatives to animal testing for the endpoints required by the REACH system (1, 2). Here, we deal specifically with an integrated, decision-tree testing strategy for skin sensitisation (as outlined previously; 3) and the alternative tests involved in the strategy.

Introduction to Skin Sensitisation

Skin sensitisation involves an irreversible change in the functioning of the immune system, caused by topical exposure to a chemical substance. Sensitisation occurs when subsequent exposure to the same, or a very similar substance (a sensitiser), leads to a greatly magnified immune reaction. Such a response can be local or systemic, and can even be lethal. It can result from contact with chemicals in consumer products or those encountered in the workplace (4). Appropriately-sensitised individuals become hypersensitive to certain subsequent skin exposures, a process known as allergic contact dermatitis (ACD). Despite the complexity of the process, and the involvement of many different cell types, much has been achieved in the last few years in defining the cellular mechanisms that are associated with, and required for, the induction of ACD due to exposure to chemicals.

The REACH Requirements for Skin Sensitisation

Under the REACH system, all chemical substances will have to be assessed for their skin sensitisation properties. The legislation states that an assessment of the available human and animal data should be carried out before performing the murine

Local Lymph Node Assay (LLNA). If the test substance is found to be corrosive, very toxic or irritant to the skin, is a strong acid (pH < 2.0) or base (pH > 11.5), or is flammable in air at room temperature, the LLNA need not be carried out, and the substance should be classified accordingly. The legislation also states that the LLNA is the preferred method for *in vivo* testing, and that a different *in vivo* test should only be used in exceptional circumstances. Justification for the use of another test would need to be provided. The other *in vivo* tests for skin sensitisation generally involve the use of guinea-pigs in either the Guinea Pig Maximisation Test (GPMT) or the Buehler test.

Outline of required tests

In the LLNA (according to OECD Test Guideline [TG] 429), the test substance is applied to the back of each ear of the mouse, and this administration is repeated each day, for three days. After two days with no treatment, phosphate-buffered saline containing ^{3}H-methyl thymidine (or ^{125}I-iododeoxyuridine and fluorodeoxyuridine) is injected into each mouse via the tail vein. After five hours, the mice are killed and the draining auricular lymph nodes from each ear are excised. A single cell suspension of lymph node cells is prepared, and the level of ^{3}H (or ^{125}I) radioactivity within the cells is determined. This test uses a minimum of four mice/dose or control group, with 16 mice typically used in a study.

In the GPMT (OECD TG 406), guinea-pigs are initially exposed to the test substance via an intradermal injection, followed by an epidermal application (the induction exposure) after 6–8 days. Following a rest period of 10 to 14 days (the induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals is compared with that in control animals. This test uses 10 animals/dose group and 5 animals/control group, with a recommended minimum of 30 animals per study.

In the Buehler test (also specified in OECD TG 406), guinea-pigs are initially exposed to the test substance by topical application, followed by further topical exposures after 6–8 days and 13–15 days, to the same area as the initial exposure. After 27–29 days, a challenge dose of the test substance is applied topically to a previously untreated area. Each dose is applied via an occlusive patch, which is held in place for 6 hours. As with the GPMT, the extent and degree of the skin reaction to the challenge exposure is compared with that of the control animals, to determine whether the test substance is a sensitiser. This test uses a minimum of 20 animals/dose group, with at least 10 animals/control group.

The guinea-pig tests measure the challenge reaction, effectively the immunological memory, whereas the LLNA is based on the measurement of the proliferative phase of sensitisation (T-lymphocyte proliferation, following the migration of Langerhans cells [LCs] to the draining lymph nodes). The LLNA is recommended for use wherever possible, as it requires fewer animals and is less invasive than the GPMT and the Buehler test. A further advantage of the LLNA (and of the Buehler test) over the GPMT, is that an adjuvant is not required. Also, unlike the guinea-pig tests, the LLNA is capable of providing a dose-response, and therefore a measure of potency.

The results obtained from LLNA tests are used to label substances according to a classification scheme for skin sensitisers: a) extreme; b) strong; c) moderate; d) weak; and e) non-sensitising (5).

The potential impact of the REACH system on numbers of tests and animals

An assessment of skin sensitisation is required for all chemicals produced or imported into the EU in quantities > 1 tonne/annum. About 30,000 chemicals are thought to be produced within this range, so a large number of animals could be needed to fulfil the testing requirements of the REACH system. However, the final REACH legislation, adopted in December 2006 (6), states that for chemicals produced/imported in quantities of between 1 and 10 tonnes/annum, new testing (including the LLNA) will only be required, if the substance is thought to be of concern (e.g. as a result of QSAR predictions, or a consideration of read-across or of existing information).

There are about 17,500 chemicals in the 1–10 tonne category (7), so the numbers of LLNA tests required in compliance with the REACH system will be dramatically less than was first envisaged. However, it is still possible that as many as 200,000 mice could be required, assuming that all the chemicals produced/imported in quantities above 10 tonnes would still need to be tested, that 16 animals would be used for each study, and that the conventional LLNA would be used.

The Available Alternative Methods

In vitro alternatives for skin sensitisation

There are three main stages in the induction of skin sensitisation by a chemical: i) penetration of the skin and reaction with skin proteins (protein binding); ii) the activation of LCs, which in turn depends on the availability and activity of certain epidermal cytokines; and iii) the stimulation of a T-lymphocyte response.

At present, there are no *in vitro* alternatives that could act as complete replacements for *in vivo* skin

sensitisation testing in compliance with the REACH system. However, several *in vitro* methods are in the course of development, and this has been facilitated by further research on the mechanisms of skin sensitisation (8–12).

Modelling Stage 1 of sensitisation

Chemical allergens (haptens) must have the ability to penetrate the epidermis and to react with specific proteins found within the skin to form stable complexes. It is thought that the hapten binds with simple protein end-groups at the recognition site of the major histocompatibility complex, class 2 (MHC II) on the cell surface of LC (13). Only a few atoms on the proteins are required, which is illustrated by the fact that the same author successfully modelled the protein-binding reaction for a range of non-sensitisers, and for weak, moderate and strong sensitisers, by using the methoxide and thiomethoxide ions as surrogates at the site of binding for activated serine and cysteine residues, respectively, (13). In addition, Roberts *et al.* (14) obtained evidence from studies on the cross-reactivity of three methylating agents, which suggested that, in skin sensitisation elicited by small haptens, antigenic specificity is directed against portions of the hapten–protein complex, rather than against the hapten itself. Further evidence for the specific involvement of only a part of the target protein has recently been provided by Aleksic *et al.* (15), who investigated the mechanism of hapten binding to human serum albumin of 10 chemicals with varying sensitising potentials. The nature of the hapten–protein complex was analysed by MS and HPLC, and it was found that protein modification, caused only by true sensitisers, was limited to certain amino acids within microenvironments that are susceptible to reactivity within the intact protein. These initial stages in skin sensitisation can be investigated by using structure–activity relationships (SARs; discussed below), although *in vitro* tests for skin penetration (OECD TG 428) and protein binding have also been developed.

Tests for protein binding are based on the fact that the majority of haptens and prohaptens are electrophilic, and will therefore react strongly with nucleophilic amino acids, such as cysteine and lysine, to form covalent bonds (16). The major types of such interactions which need to be considered for skin sensitisation, are: Michael-type reactions; S_N2 reactions; S_NAr reactions; acylation reactions; and Schiff-base formation (17). Roberts and Williams (18) suggested the use of a Relative Alkylation Index (RAI) for comparing the potencies of different sensitisers on the basis of their electrophilic interactions with protein. The RAI depends on the relative degree of covalent binding (alkylation) to protein which occurs at induction and challenge, and was derived on the basis of the binding reaction in a hydrophobic environment and the removal of the sensitiser by its partitioning into polar lymphatic fluid, as in the LLNA.

Some chemicals require metabolic activation in order to react with protein, and these are called prohaptens. The importance of bioactivation for protein binding is illustrated by reference to the work of Berl *et al*. (19), who concluded that the sensitising potential of the drug, propacetamol, in human patch testing was due to its conversion to *N,N´*-diethylglycine, which is able to undergo alkyl transfer to proteins in the skin.

The concept of protein binding was used by Gerberick *et al*. (20) to develop an assay based on binding to cysteine-containing glutathione (GSH) and to three synthetic peptides, all containing either cysteine, lysine or histidine. This assay was used to assess the reactivities of 38 chemicals with different sensitising potentials, ranging from extreme sensitisers to non-sensitisers, via the measurement of GSH depletion and of binding to the synthetic peptides. The results suggested that the assay has the potential to be used as an initial screen in skin sensitisation testing, as part of a testing strategy. Aptula *et al*. (21) used a similar glutathione assay, in conjunction with a further *in vitro* test for toxic potency, to correctly predict the different *in vivo* sensitising potentials of 23 out of 24 chemicals. Binding was measured via a thiol reactivity index, based on reduced GSH. However, these authors cautioned that, due to the selectivity of electro(nucleo)philic reactions, some sensitising chemicals would be missed when a single nucleophile such as thiol, was used to measure electrophilic binding.

Modelling Stage 2 of sensitisation

The second stage of sensitisation involves LCs, which are specific dendritic cells (DCs) found in the epidermis. These cells are also found in lymphoid organs, and are primarily responsible for interacting with, as well as processing and transporting, antigenically-active substances present in the skin (22). Following topical sensitisation, LCs at the site of exposure are induced to leave the epidermis and travel to the draining lymph nodes, during which time they acquire immuno-stimulatory properties which enable them to present antigens to responsive T-lymphocytes (23). The processes of sensitisation are mediated by specific cytokines and other factors, including granulocyte/macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), the co-stimulatory molecule, CD86, and the intercellular adhesion molecule, CD54 (24–28).

The complexity of the process has been a major reason for the slow development of *in vitro* methods

for skin sensitisation (for reviews, see 27–29). The biggest problem with using LCs *in vitro*, is obtaining a sufficient number of cells for routine use. Therefore, research has been focused on other forms of immunologically-active cells, such as those derived from monocyte DCs, which can readily be obtained from the bone-marrow and from umbilical cord blood, and then analysed for changes in cytokine production following exposure to potential and known sensitisers (29). Such sources of human monocyte DCs can be used individually to give donor-specific results, or pooled to provide more-generalised data (see 30).

The functional maturation of DCs in response to sensitisers is associated with the altered expression of a large number of cytokines, chemokines, cytokine and chemokine receptors, co-stimulatory molecules, and many other gene products. This was first described by Enk and Katz (31), who observed that the topical application of contact allergens to mice, stimulated an increase in the expression of IL-1β mRNA. A corresponding experiment with skin irritants did not stimulate an increase in IL-1β expression (the ability of sensitisation tests to distinguish irritants from sensitisers is crucial). Further studies on human-derived DCs have shown similar results, although there is often a lack of sensitivity, even when known potent sensitisers are used (29).

Microarray and proteomics investigations have been used to study changes in gene expression and protein production in response to skin sensitisers. It is clear that such methodology could also be applied to assays based on detecting changes in IL-1β mRNA expression by DCs, and for the transcript analysis of contact allergen-induced DC differentiation (32, 33).

An example of a focused study on toxicogenomics, as applied to sensitisation, is the recent investigation by Schoeters *et al*. (34), who used cDNA microarrays to assess the transcriptional activity patterns of 11,000 human genes in human DCs derived from $CD34^+$ progenitor cells exposed to either the sensitiser, dinitrochlorobenzene (DNCB), or to solvent, for 3, 6 and 12 hours. Compared to the gene expression of the controls, changes larger than about two-fold were observed for 241 genes after exposure to DNCB, of which 137 were up-regulated and were 104 down-regulated. Interestingly, 20 of the genes were known to encode proteins that are related to the immune response. A further study, by Gildea *et al*. (35), involved a similar type of analysis of 10 genes (from peripheral blood-derived DCs), the expression of which demonstrated reproducibly high levels of selectivity and specificity for skin-sensitising chemicals. These authors foresee the use of a panel of these genes to phenotype the activities of test chemicals.

Hirota and Moro (36) used micorarrays to discover that a novel biomarker for sensitisation, MIP-1β, was up-regulated when THP-1 cells (a human monocytic leukaemia cell line) were exposed to known skin sensitisers, such as 2,4-dinitrochlorobenzene, *p*-phenylenediamine, and nickel sulphate. In contrast, no effects were observed with non-sensitisers, such as the irritants, sodium dodecyl sulphate (SDS) and benzalkonium chloride. When MIP-1β production and CD86 expression were measured, results largely in agreement with *in vivo* data were obtained in this system.

THP-1 cells have the advantage over human-derived DCs that they do not show donor-specific differences. Yoshida *et al*. (37) found that naïve, as opposed to cytokine-treated, THP-1 cells were more sensitive to the induction of CD54 and CD86 expression by known sensitisers. After using the assay to assess the activities of a range of chemicals, it was observed that known sensitisers induced CD54 and CD86 expression in a concentration-dependent manner, while non-sensitisers were inactive. Ashikaga *et al*. (38) have subsequently optimised this assay, and used it as a basis for developing the human-Cell Line Activation Test (h-CLAT). In a collaborative study in two separate laboratories (39), this test exhibited high inter-laboratory agreement, and a multi-laboratory study is planned to further optimise the protocol. CD86 induction has also proved to be a useful marker of sensitisation in MUTZ-3 cells (a cytokine-dependent human monocytic cell line), which are even responsive to mild sensitisers such as benzocaine, which can be difficult to detect in the LLNA (40).

Research is also being undertaken to develop more-complex *in vitro* assays for skin sensitisation, including reconstituted skin models containing functional DCs (41–43). Such organotypic models should provide information on human sensitisation of increased relevance for risk assessment, as they possess some barrier function. Facy *et al*. (44) used human $CD34^+$-derived LCs (from cord blood) that have been incorporated into the EPISKIN construct (www.invitroskin.com), with detection of IL-1β up-regulation and CD86 expression as endpoints. As with the single DC culture systems, pooled or individual donor $CD34^+$ LCs can be used. The effects of benzocaine were also detected in this organotypic skin model system.

Modelling Stage 3 of sensitisation

Stage 3 of sensitisation involves the activation of T-lymphocytes by the antigen (presented by the activated LCs), with concomitant cell proliferation and the production of allergen-specific T-lymphocytes. The quantitative increase in T-cells capable of recognising and responding to the inducing allergen, represents the cellular basis of sensitisation. Efforts to develop *in vitro* techniques for this stage of sensitisation have been less successful than those

for the earlier stages. A key problem is that stimulation of T-lymphocytes *in vitro* requires the presence of specifically primed T-lymphocytes from previously-sensitised animals (45, 46). At present, there is no direct means of obtaining T-lymphocytes that have been pre-sensitised to specific test substances. However, recent attempts to use DCs, modified with specific haptens, to provoke T-lymphocyte proliferative responses *in vitro*, have had some success. Rustemeyer *et al.* (47) reported the development and application of a protocol for hapten-specific T-cell priming, whereby DCs (generated from peripheral blood mononuclear cells) were successfully used to sensitise autologous naïve T-cells to two common contact sensitising agents, nickel sulphate and 2-hydroxyethyl-methacrylate. When challenged with haptens not used in the T-cell priming, no response was observed.

Results obtained by Dai and Streilein (48) showed that T-cells taken from normal human blood can be induced to develop into hapten-specific T-cells (by using a similar method to that described above) that closely resemble those taken from hapten-immune donors (by using DNCB). This suggests that the technique could be useful as a test method.

Finally, Guironnet *et al.* (49) developed an assay which uses monocyte-derived DCs to analyse the autologous proliferative T-cell response to allergens. When DCs that had been treated with a known allergen, trinitrophenyl, were assayed, a significant T-cell proliferative response was observed, whereas when the irritant, SDS, was used, no proliferative response was apparent. This suggests that the method could be useful for discriminating between strong contact sensitisers and irritants.

In response to the need for *in vitro* methods for skin sensitisation required by the REACH legislation and the 7th Amendment to the Cosmetics Directive (50), an EU-integrated project has been established, known as *Sens-it-iv* (www.sens-it-iv.eu). The aim of this project is to actively encourage research and collaboration in this area, with the aim of developing *in vitro* assays and testing strategies to replace animal studies for both skin and respiratory sensitisation.

The *in silico* prediction of skin sensitisation

Barratt (51), Lepoittevin (52), Cronin *et al.* (53), Rodford *et al.* (54), and an OECD expert group (55) have all reviewed the use of quantitative SAR (QSAR) modelling for skin sensitisation. The reader is also referred to the review by Dupuis and Benezra (56), which remains a definitive source of information on the chemical basis of skin sensitisation, and to two papers by Roberts *et al.* (57, 58), which provide a detailed discussion of the use of electrophilic chemistry as a basis for predicting sensitisation. Benezra *et al.* (59) described the development of a database containing several thousand results of contact dermatitis tests, together with a structural classification scheme (called the "Structure–Activity Tree").

Good examples of the *in silico* approach include: Sosted *et al.* (60); Patlewicz *et al.* (61, 62), Roberts and Basketter (63), Hostýnek *et al.* (64, 65), Mekenyan *et al.* (66), and Ashby *et al.* (67). Also, individual QSARs, based on some specific chemical classes, are shown in Table 1. The majority of these studies either relate some parameter modelling dermal partitioning (e.g. log P) to potency, and assume that reactivity is constant, or they incorporate some description of reactivity, such as a molecular orbital property or structural feature. Several studies have also attempted to model skin sensitisation for large databases (68–70), but success has been limited.

Several expert systems exist for predicting skin sensitisation. TOPKAT is based on GPMT data (71), and MultiCASE is based on human sensitisation data (72). Hostýnek and Maibach (73) have also reviewed the application of *in silico* approaches for predicting ACD, and in particular, discussed the use of BIOSAR models, based on statistical analysis with chemical knowledge in the form of expert systems (see also, 74).

The OASIS/TIMES (TIssue MEtabolism Simulator) models are based on various types of data, and include a module for predicting metabolic activation, to take account of the existence of prohaptens that need to be activated before they can penetrate the skin and react with protein (75). In this respect, Dimitrov *et al.* (76) described a SAR/QSAR system for estimating skin sensitisation potency, based on the joint prediction of skin metabolism (by using TIMES), and the potential of parent chemicals and/or their activated metabolites, to react with skin proteins. The resulting SAR/QSAR system correctly classified about 80% of the chemicals with significant sensitising effect, and 72% of non-sensitising chemicals. More recently, TIMES has been the subject of a comprehensive review of its ability to predict skin sensitisation (77).

DEREK now has an extensive rulebase for sensitisation, following on from the work of Payne and Walsh (78), which includes over 60 rules for this endpoint, some of which have been subjected to a validation study (79, 80). DEREK is the most widely-used expert system for predicting sensitising potential. Its rulebase has recently been updated, to take account of enhancements to toxicophore definition, mechanistic classification, and the extent of supporting evidence provided for a range of aldehydes, 1,2-diketones and isothiazolinones (81).

DEREK and TOPKAT have been compared for their predictivities of skin sensitisation, along with an original method based on logistic regression methodology (82). This study found that correct classifications were given for guinea-pig data for 73.3%, 82.9% and 87.6% of the time by TOPKAT, DEREK, and the

Table 1: Examples of individual QSARs for skin sensitisation, based on specific chemical classes

Chemical class	Descriptor(s)	Reference
Hydrophobic (log P ≥ 5) sulphonate esters:		
LLNA data	Negative hydrophobicity co-efficient	(104)
Guinea Pig data	Electrophilicity, hydrophobicity	(105)
Aldehydes (containing a benzene ring)	Electrophilic reactivity	(106)
Chemicals capable of acting via Schiff-base formation	Reactivity, Log P	(107)
Carbonyl-containing chemicals	Electrophilicity, Log P	(108)
3-substituted catechols	Extent of carrier haptenation	(109)
Bromoalkanes	Hydrophobicity	(110)
Anthraquinone dyes	Hammett sigma constants, energy gap between HOMO and LUMO	(111)
Phenyl benzoates	Molecular volume, calculated Log P	(112)
Small molecular weight fragrance allergens	Transport/binding, Log P, polarisable molecular volume, H-bond acceptor/donor properties, protein reactive substructures	(113)
α,α-dimethyl-γ-butyrolactone derivatives	Log P, measured relative rate constants for reactions of the lactones with *n*-butylamine	(114)

logistic regression model, respectively. In addition, the authors found that correct classifications were given for LLNA data for 73.0% and 83.2% of the time by DEREK and the logistic regression model, respectively. Lastly, Anderson *et al*. (83) have used a combination of TOPKAT, DEREK and another QSAR-based expert system (NIOSH Logistic Regression) to predict the contact and respiratory sensitisation potentials of a range of volatile oxygenated organic chemicals, with the results being compatible with data obtained from the LLNA. DEREK has also been used in combination with another modelling approach, called TOPS–MODE (84).

The reaction mechanistic domain approach to in silico *and* in chemico *prediction of skin sensitisation*

It was argued in a recent review of the various stages in the skin sensitisation process (85) that the ability of a substance to bind covalently to carrier protein is the key factor which determines sensitisation potential, and that, for the prediction of skin sensitisation potential, this is the process which needs to be modelled and the area where more research effort should be focused. Elsewhere, it has been argued that chemicals can be classified into a limited number of reaction mechanistic domains (86). Furthermore, it was suggested that, within these domains, QSARs or Quantitative Mechanistic Models (QMMs) can, in principle, be derived, based on the RAI model, which relate sensitising potential to a combination of electrophilic reactivity and hydrophobicity. In line with these mechanistic principles, the following sequential strategy was proposed when a new chemical is presented (85–87):

1. Classify the chemical into its reaction mechanistic domain. One domain is the "unreactive" domain, populated by predicted non-sensitisers. For several mechanistic domains, there are corresponding pro-electrophilic sub-domains. For example, many sensitisers, such as hydroquinone and 3-alkyl/alkenyl catechols, are thought to act as pro-Michael acceptors. Domain classification can often be made possible by the inspection of structure, but inevitably in some cases, a confident prediction may not be possible. In such situations, experimental work will be

needed to determine the reaction chemistry, especially to determine whether the compound is electrophilic or pro-electrophilic, and the nature of the reactions.

2. Quantify the reactivity/hydrophobicity of the chemical relative to known sensitisers in the same mechanistic applicability domain. It might be possible to predict these properties from structure, by using physical organic chemistry approaches, such as linear free energy relationships based on substituent constants, or on molecular orbital parameters. In other cases, it could be necessary to perform physical organic chemistry measurements, such as the determination of reaction kinetics and the measurement of partition coefficients.

3. Undertake QSAR, QMM or mechanistic read-across to predict the sensitisation potential.

Read-across and the OECD QSAR Application Toolbox

Read-across is a technique in which analogues, or members of the same class of compounds, are assumed to have the same (for a qualitative endpoint) or similar (for a quantitative endpoint) activities. These techniques are suitable for interpolating activity, when appropriate data are available. A crucial first step in applying read-across is the identification of classes or categories of chemicals that are sufficiently similar physico–chemically to have similar biological effects. With regard to skin sensitisation, read-across is applicable, in principle, on the basis of mechanistic classes such as those described above. At the time of writing, a useful tool for applying read-across is being developed by the OECD as part of a QSAR Application Toolbox. It is intended to facilitate the identification of suitable categories of chemicals and the application of read-across for a wide variety of endpoints, including skin sensitisation. The toolbox has been designed to enable clear and mechanistically-transparent predictions to be made, and will become freely available in 2008.

Opportunities for Reducing and Refining the Use of Animals

Steiling *et al*. (88) made a series of suggestions for improving and simplifying the OECD-recommended tests for skin sensitisation, including: a) the halving of group sizes; b) the possibility for needing only one positive control with the sharing of data; c) the importance of obtaining re-challenge data; d) the redundancy of pre-treating with SDS in the GPMT; and e) the use of the LLNA as a replacement for the guinea-pig tests.

The LLNA was incorporated into the OECD Health Effects Test Guidelines in 2002, after being validated and endorsed by both ECVAM (89) and ICCVAM (90). The test has the following important welfare and scientific advantages: a) it requires no intradermal injections of adjuvant or test material (less invasive); b) fewer animals are used than in the guinea-pig tests; b) no severe skin reactions are involved; c) quantitative potency information is provided; and d) it is a relatively rapid test, requiring five days for the *in vivo* phase, as compared with 30 days for the GPMT and Buehler tests.

Further reductions in the number of animals used for the LLNA test are possible, by relying on the use of pre-existing control data. In the first case, it would be possible to eliminate the need for positive controls, thus reducing the number of animals needed per test by at least 4. This is suggested in OECD TG 429 (91), subject to the updating of historical positive control data every six months. The TG also states that, where possible, dermal irritancy and acute toxicity data should be taken into account, to determine dose ranges. These reduction measures should be further evaluated and, subject to their workability, made mandatory.

A further modification of the LLNA, the reduced LLNA (rLLNA), has recently been suggested, and involves administering only what would have been the top dose in the normal version of the test (92). An existing LLNA database of 211 chemicals was analysed, to determine whether the rLLNA protocol (involving a single high-dose group and a concurrent vehicle control) would have been sufficient for predicting the sensitising potential in the context of a testing strategy. It was concluded that the rLLNA approach was justifiable for the dataset analysed, and an independent peer review by the ESAC has recently endorsed the approach for screening chemicals in relation to, for example, the REACH legislation. It should be noted that the rLLNA is not suitable for a full quantitative risk assessment, as the data obtained do not permit the determination of sensitising potency. However, where the application concentration is above a threshold of 10%, it is deemed that the rLLNA is able to screen for sensitisation, as it has generated no false positives and few false negatives. Nevertheless, if a full quantitative risk assessment is required, then the full LLNA (including a range of dose levels to determine potency) has to be used.

The overall predictive performance of the LLNA is at least as good as that of the guinea-pig tests, particularly for distinguishing between strong and moderate sensitisers. There are a few cases, however, where the LLNA may not be applicable; for example, for: a) metallic compounds (e.g. metals, metal salts and organometallic materials); b) runny liquids and wholly aqueous vehicles, when they do not adhere to the ear sufficiently; and c)

some strong dermal irritants, which might produce false positive results.

When the LLNA is considered unsuitable, the Buehler test (OECD TG 406) should be used in preference to the GPMT, as it involves the topical application of test substance, instead of intra-dermal injections, and does not require an adjuvant. Lastly, a further refinement of the GP methods would be to train the animals for use with the required restraining apparatus (93).

Integrated Testing Strategies for Skin Sensitisation

Recently-proposed testing strategies

A stepwise process for the determination of skin sensitisation was proposed by an ECVAM working group (94), which included: a) an assessment of historical data; b) an assessment of physico–chemical properties; c) the screening of structures by using the DEREK skin sensitisation rulebase; d) an assessment of partition parameters; e) the *in vitro* assessment of skin sensitisation; and f) a LLNA, if necessary. This scheme was based on a hierarchical testing strategy, originally developed by Unilever, that requires the empirical determination of skin penetration *in vitro* (95), in conjunction with a DEREK prediction of structural alerts (96).

The BUAV (97) proposed an animal-free testing strategy, which initially involves an *in silico* prediction with DEREK, followed by a validated *in vitro* skin penetration study on skin fragments, an *in vitro* test to see whether the test substance reacts with human serum proteins, and, finally, more *in vitro* tests involving the use of LCs and DCs, depending on the results obtained within the scheme. This strategy requires the use of several key tests that have yet to be validated, and, while possibly being suitable for classification and labelling, its use for regulatory risk assessment would be considered controversial by many.

More recently, Jowsey *et al.* (25) have proposed a new approach to testing skin sensitisation, which is based on measuring five biological and physico–chemical processes associated with skin sensitisation. These are: a) structural alerts (from QSAR and/or expert systems); b) bioavailability (based on molecular size, net electrical charge and log P); c) likely protein reactivity; d) impact on the function of DCs or DC-like cells; and e) induction of a T-lymphocyte response. Each of these phenomena is rated according to the results obtained for a given test substance. The product of these scores is then used to determine an Index of Sensitising Potency (ISP) value, where a higher score indicates a higher sensitising potency.

Our suggested decision-tree integrated testing scheme for skin sensitisation

Our own strategy has been developed as a decision-tree, whereby a decision can be made at various steps as to whether sufficient relevant information is available to justify the cessation of testing, so that classification and labelling and/or risk assessment can be performed. The first step requires all the relevant existing data from animals and humans to be collected and analysed, before any testing is conducted. At this point, it may be decided that the information can be used for classification and labelling and/or risk assessment, so no new testing is required. The strategy also includes a weight-of-evidence evaluation, whereby all the data collated, and the results from all the tests completed so far (*in silico*, *in vitro*, and 'in house') are evaluated, to determine whether any animal testing is actually necessary. These steps are very important, if the number of animals which could be used to fulfil the REACH requirements, is to be minimised.

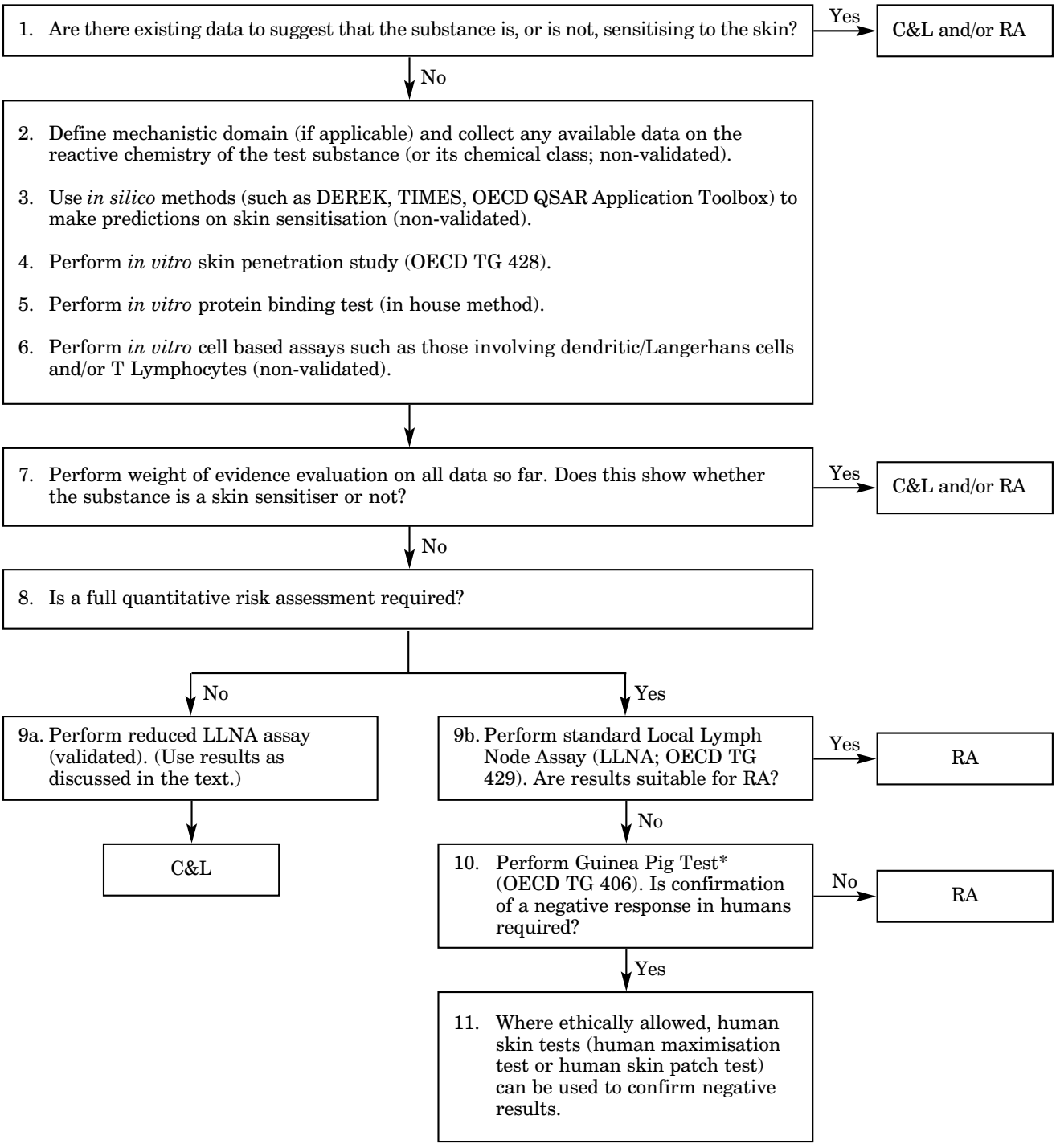
Each test method proposed within the strategy has its validation status described as either: *non-validated* — tests which are generally still undergoing research and development, and are unlikely to be available for regulatory use in the short-term; *being validated* — tests for which pre-validation or full validation studies are on-going, and which may be available for regulatory use in the short-term; or *validated* — tests which have successfully been validated in a study conducted according to internationally agreed criteria, but which have yet to be granted regulatory and/or OECD test guideline (TG) status. Each test which has been validated and approved by the OECD, is shown with its respective OECD TG number.

Tests described as 'in house' are those which are undertaken alongside non-animal tests, and can aid decision-making with regard to regulatory approval. These tests can help to decipher the information — such as mechanisms of toxic action or metabolism profiles — from *in silico* and *in vitro* results, but they are not, themselves, likely ever to be used as full or partial replacements for *in vivo* test methods.

The skin sensitisation testing strategy

There are ten steps in the proposed testing strategy for skin sensitisation (Figure 1), two of which involve the use of animals, and four of which involve decision-points. Prior information for Step 1 could come from skin sensitisation studies and from eye or skin corrosion/irritation studies (e.g. it is known that individuals with hypersusceptibility to irritation are more likely to exhibit ACD than are those with normal susceptibility [98]). Positive data

Figure 1: An integrated testing strategy for skin sensitisation



**We recommend the use of the Buehler Test over the GPMT, as it is less invasive. C&L = classification and labelling; RA = risk assessment.*

from genotoxicity studies might indicate a potential for reacting with nucleophilic target molecules (e.g. DNA, in this case, acting as a surrogate for protein), to see whether the substance is an electrophile, and by implication that it could bind to immunological proteins in the skin. Occupational exposure and epidemiological studies could also provide useful information at this stage.

Step 2 involves defining the applicability domain and the chemical reactivity of a test substance, followed by Step 3, which involves the *in silico* prediction of sensitisation potential, by using one or more

of the available QSAR models and expert systems. However, *in silico* prediction by itself is not considered adequate to obviate the need for any further testing. Thus, other properties of the test substance are evaluated in Steps 4–6 (chemical reactivity; *in vitro* skin penetration; *in vitro* protein binding; *in vitro* cell-based assays, e.g. with DCs/LCs and/or T-lymphocytes).

Information from all the above tests is used in a weight-of-evidence evaluation in Step 7. Step 8 poses the question of whether a full quantitative risk assessment is required, as there are two different routes for Step 9, depending on the final requirements of the strategy. Step 9a involves the use of the rLLNA. This assay is not suitable, if a full risk assessment is required, as it does not give potency data. It is only used as a screen and for classification and labelling purposes. If this branch of the testing strategy is used, no further testing is required.

If a quantitative risk assessment is required, then the opposite branch of the testing strategy is used. The full LLNA is performed in Step 9b. If equivocal results are obtained in the LLNA, or the nature of the test substance means that the LLNA is inappropriate, then use of the GPMT or Buehler tests in Step 10 could be considered, but only in very extreme circumstances, and as a last resort. The Buehler test is preferred, as it is less invasive than the GPMT.

Lastly, it might be appropriate and useful, when permissible according to local ethical regulations, to perform skin tests on human volunteers (Step 11), such as the human maximisation or human skin patch tests, in order to confirm the identification of negative sensitisers (99). The decision to conduct such tests needs to be taken with great care, in view of the possibility of sensitising an individual as a result of the testing (100).

Conclusions

The first part of our proposed decision-tree integrated testing strategy is based on the substantial achievements that have been made over the last few years, in identifying the molecular and cellular mechanisms associated with, and required for, the induction of sensitisation by chemicals. We now have detailed information on the processes by which potential sensitisers interact with skin proteins to elicit the very first stages of sensitisation. This information has been used, together with other data on the activities of a wide range of chemicals in animal tests for sensitisation, to enable the formulation of various QSAR models and expert system rules for the *in silico* prediction of skin sensitisation.

In addition, the development of *in vitro* systems for sensitisation testing has been facilitated by advances in the co-culture of immunologically-active cells, a greater understanding of the roles of these cells in the immune response, and the ability to measure the production of relevant and specific cytokines in response to the challenging of target cells with allergens.

The second part of the testing strategy is focused on the use of the LLNA, initially as a reduced version for screening (the rLLNA), and, later in its original form, with the use of traditional guinea-pig tests (preferably the Buehler test), only in exceptional circumstances, as a final resort. The rLLNA has been endorsed for chemicals screening, but we consider that it would be rare for a company to need to screen a large number of chemicals for the purposes of the REACH system. It is more likely that a full individual risk assessment for each chemical would be required, in which case, the full LLNA would need to be undertaken, to avoid the need to use both versions of this animal test. We envisage three scenarios in which the rLLNA could be considered: a) when a company has several chemicals fulfilling the same function; b) during the development of an alternative safer chemical, when several candidates need to be tested; and c) if only classification and labelling are required (i.e. no risk assessment is needed). The rLLNA might also be used to determine whether an *in vitro* result is a false-negative.

Our scheme does not include any assays based on genomic analysis, because these approaches are still at the early stages of development, and are not yet ready for use in routine regulatory testing. Nevertheless, they have great promise (101), and have already been used to provide useful information in focused studies.

Nevertheless, despite all the advances that have been made in understanding sensitisation, and the improvements to methods for identifying sensitisers, the complete replacement of animal testing for this endpoint remains elusive. This will depend on further developments, to allow: a) improved QSAR and expert system modelling; b) the validation of such models; c) *in vitro* modelling of the highly-complex process of antigen presentation (see 102); d) the incorporation of biotransformation systems into subcellular and cellular models of sensitisation; e) the use of organotypic human skin model systems with realistic barrier function; and f) the use of *in vitro* data for risk assessment (103).

In the meantime, we believe that the application of a testing strategy, such as the one presented in this paper, should be used, particularly for the purposes of satisfying the EU REACH legislation for testing chemicals for skin sensitisation.

Recommendations

1. Improvements need to be made to existing rule-bases for the expert system prediction of sensiti-

sation, and particularly to allow for the combined used of metabolism and sensitisation prediction software.

2. New QSARs for sensitisation need to be developed, to widen the overall applicability domain of *in silico* prediction methods. In particular, a large database needs to be established, comprising experimental reactivity data (ideally including information on binding kinetics) for compounds for which LLNA potency data are already available, with good coverage of all the major domains (Michael acceptors, S_N2 electrophiles, S_NAr electrophiles, Schiff-base formers, and acyl transfer agents). This database could then be used to develop QSARs and QMMs, with wider domains suitable for read-across.

3. *In silico* prediction methods need to be validated according to the internationally-recognised validation criteria.

4. Methodology needs to be developed for the inclusion of biotransformation systems in subcellular and cellular tests for sensitisers.

5. Further investigations into the cellular basis of sensitisation need to be undertaken, to provide information that will allow all the critical stages of the immune response to be successfully modelled *in vitro*.

6. The use of genomic methodologies, to permit the application of toxicogenomic approaches to sensitisation testing, needs to be expedited.

7. An OECD TG for the rLLNA needs to be written, and a Guidance Document should be prepared, to assist users in choosing between this and the full version of the test.

8. Where guinea-pig tests are required in compliance with the EU REACH legislation, it should be clearly stated that the use of the Buehler Test is preferred to the GPMT, for scientific and welfare reasons. This distinction would be made easier, if the OECD were to produce two separate TGs for these methods.

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