

Non-animal test methods for predicting skin sensitization potentials

Annette Mehling · Tove Eriksson · Tobias Eltze ·
Susanne Kolle · Tzutzy Ramirez · Wera Teubner ·
Bennard van Ravenzwaay · Robert Landsiedel

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Abstract Contact allergies are complex diseases, and it is estimated that 15–20 % of the general population suffers from contact allergy, with increasing prevalence. Evaluation of the sensitization potential of a substance is usually carried out in animal models. Nowadays, there is much interest in reducing and ultimately replacing current animal tests. Furthermore, as of 2013, the EU has posed a ban on animal testing of cosmetic ingredients that includes skin sensitization. Therefore, predictive and robust *in vitro* tests are urgently needed. In order to establish alternatives to animal testing, the *in vitro* tests must mimic the very complex interactions between the sensitizing chemical and the different parts of the immune system. This review article summarizes recent efforts to develop *in vitro* tests for predicting skin sensitizers. Cell-based assays, *in chemico* methods and, to a lesser extent, *in silico* methods are presented together with a discussion of their current status. With considerable progress having been achieved during the last years, the rationale today is that data from different non-animal test methods will have to be combined in order to obtain reliable hazard and potency information on potential skin sensitizers.

Keywords Skin sensitization · Contact allergy · Alternative methods · *In vitro* testing · 3Rs principle

Introduction

Topically applied substances, in particular various, predominantly electrophilic, low-molecular-weight (LMW) substances, have the potential to give rise to allergic reactions in susceptible individuals (Nguyen et al. 2008). It is therefore of great importance for the chemical, pharmaceutical and cosmetic industries to identify potential sensitizers already at an early stage. The sensitizing potential of chemical substances is usually evaluated in animal tests, such as the murine local lymph node assay (LLNA; OECD TG 429) or the guinea pig-based tests described in OECD TG 406. However, there is an increasing interest from the public as well as from regulators for reducing and ultimately replacing animal tests. Within the European Union (EU), a testing and marketing ban has been implemented in the Cosmetics Directive (76/768/EEC), now Cosmetics Regulation (EU 1223/2009), covering animal tests to evaluate the human safety of cosmetic products and their ingredients. In accordance with the 7th Amendment (2003/15/EC) to the Cosmetics Directive, in the EU, animal testing of finished cosmetic formulations has already been banned since 2004, and a further ban on cosmetic ingredients and products tested on animals has been imposed from 2009 on. This testing ban is accompanied by a concomitant marketing ban with the exception of ingredients tested for repeated-dose toxicity, reproductive toxicity and toxicokinetics. According to the current legislative status, the full marketing ban for any ingredient tested on animals for the purpose of the Cosmetics Directive regardless of the availability of non-animal

A. Mehling
BASF Personal Care and Nutrition GmbH, Düsseldorf, Germany

T. Eriksson
Sten stures väg 69, 131 47 Nacka, Sweden

T. Eltze · S. Kolle · T. Ramirez · B. van Ravenzwaay ·
R. Landsiedel (✉)
BASF SE, Experimental Toxicology and Ecology,
GV/TB, Z470, 67056 Ludwigshafen, Germany
e-mail: robert.landsiedel@basf.com

W. Teubner
BASF Schweiz AG, Basel, Switzerland

alternative test methods will come into force in March 2013. To date, there is no non-animal method for skin sensitization testing having gained full regulatory acceptance. Therefore, predictive and robust alternative methods to replace the currently used animal tests are urgently required.

The concern about animal testing is not a new phenomenon. The 3Rs concept, Replacement, Reduction and Refinement, was first described by Russell and Burch in (1959) with the aim to foster the development of alternative methods. The European Centre for the Validation of Alternative Methods (ECVAM) was set up in 1991 in order to promote the development and validation of 3Rs methods, which also is the intention of the United States Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). In vitro alternatives to animal testing should be accurate, reliable, robust and, last but not least, the tests must be predictive of effects observed in humans. To fully replace the current animal models, in vitro models must not only identify the hazard of substances, but also be able to predict their relative potency. This is especially important for skin sensitizers, since their potency is believed to vary up to five orders of magnitude. Furthermore, a substantial part of the known contact allergens requires some kind of activation in the skin in order to act as potent allergens. In vitro testing strategies must take this fact into consideration (Kimber et al. 2011). Another challenge is that assays should be sufficiently sensitive to enable the detection of weak allergens. During the last decades, extensive work has been conducted in developing in vitro assays to replace current animal test methods for the predictive identification of skin sensitizers. This research has been carried out by a number of groups, including Cosmetics Europe—the Personal Care Association (previously known as COLIPA)—and the Sens-it-iv consortium as well as by various companies and universities. Sens-it-iv was an integrated EU-funded research project involving 28 partners, whereof 9 represented industry and 15 universities or research institutes. The further 4 partners were the European Commission Joint Research Centre (JRC), Cosmetics Europe, the In Vitro Testing Industrial Platform (IVTIP) and the European Consensus Platform for Alternatives (ECOPA). The Sens-it-iv project began in 2005 and was finalized in March 2011. Its goal was to develop methods and/or alternative strategies to animal testing for the prediction of potential skin and respiratory sensitizers. Substantial research has also been supported directly by Cosmetics Europe. Both fundamental research and applied research have been funded in areas such as the modeling of skin bioavailability, hapten chemistry, peptide binding, skin metabolism, dendritic cell activation and T-cell proliferation, all aiming to develop predictive in vitro methods for

skin sensitization hazard (Aeby et al. 2010). In addition to the efforts carried out by Sens-it-iv and Cosmetics Europe, considerable work has also been accomplished by individual companies as well as within academia. Complementary to the experimental work that continues to be performed, a joint platform for stakeholders, the European Partnership for Alternative Approaches to Animal Testing (EPAA), has been formed in the EU with the aim to facilitate and accelerate the establishment of alternative methods.

Before any substance (pharmaceutical, cosmetic, chemical, etc.) can be brought into the European market, it has to be evaluated in regard to its safety to humans and the environment. The European chemicals legislation on the Registration, Evaluation and Authorization of Chemicals (REACH; Regulation (EC) No 1907/2006) foresees evaluating an estimated 30,000 chemicals by the year 2018, many of which will have to be tested for hazardous effects (EC 1907/2006). Skin sensitization is an endpoint described in Annex VII of the REACH regulation, which implies that its evaluation is mandatory for all REACH-relevant substances manufactured or imported in quantities of one ton or more per year. The final assessment of a REACH registration dossier should enable determining whether a substance is a skin sensitizer or not. The REACH information requirements state that assessment of all available human, animal and non-animal data has to be taken into account before initiating any in vivo testing. Furthermore, it is clearly laid down that, if an in vivo method is employed, the LLNA is the first choice method for testing the sensitization potential of substances. Only in exceptional cases should other animal tests (e.g., guinea pig maximization or Buehler tests, OECD TG 406) be conducted, and selection of these latter tests requires specific justification. Importantly, the REACH regulation allows using non-animal test methods, such as computational models and in vitro assays, again provided that their choice is based on a robust scientific justification. Classifications based on these methods generally result in yes-or-no answers and do not yield information on potency. Until now, this has been considered sufficient for regulatory classifications. Recently, however, the amendment to the Regulation on the Classification, Labeling and Packaging of Substances and Mixtures (CLP; Regulation (EC) No 1272/2008), the European implementation of the Globally Harmonized System of Classification and Labeling (GHS), which applies to single substances since December 1, 2010, and will apply to mixtures as of June 1, 2015, foresees classifying allergens into either category 1A (strong sensitizers) or category 1B (other skin sensitizers). Since the in vitro methods under development have predominantly focused on assessing the sensitizing hazard of a substance, and not its potency, the new classification scheme will prove challenging.

Starting out with an overview on the pathophysiological process of sensitization and on available *in vivo* methods, this review summarizes the current status of *in vitro* methods, in chemico and, to some extent, in silico methods for predicting the skin sensitization potential of substances, taking into account the results obtained in the above-mentioned scientific initiatives and focusing on the relevance of non-animal test methods for regulatory purposes and for meeting the goals of the 3Rs principle to refine, reduce and ultimately replace animal testing.

Sensitization

Contact allergies are complex diseases, and it is estimated that 15–20 % of the general population will suffer from contact allergy at some point in life, with most likely increasing prevalence. Important risk factors for developing allergic contact dermatitis (ACD) are workplace, exposure, age, sex, use of consumer products and genetic predisposition (Nguyen et al. 2008; Peiser et al. 2012). Primarily due to high exposure, the most commonly reported causes of ACD are nickel, fragrances, preservatives and hair dyes (Thyssen et al. 2007). The high prevalence of allergic diseases not only results in large costs for the healthcare system, but also greatly impairs the quality of life of the affected patients.

ACD resulting from type IV, cell-mediated hypersensitivity reactions is the clinical manifestation of sensitization induced by skin contact with LMW allergens often referred to as haptens. This type of hypersensitivity response develops in two phases, the induction or sensitization phase, during which the immune system is sensitized, and the elicitation or challenge phase, during which the symptoms develop (Fig. 1). In the *in vivo* situation, the substance must first penetrate through the stratum corneum of the skin. For a hapten to elicit an immune response, it must be able to react by covalent binding with endogenous proteins present in the skin. During the sensitization phase, immature dendritic cells (DCs) of the skin, so-called Langerhans cells (LCs), internalize the hapten or hapten/protein complex. If danger signals, for example inflammatory cytokines, such as interleukins (IL) and tumor necrosis factor (TNF)- α , are secreted into the tissue by keratinocytes as a response to trauma, the immature DCs become activated and mature. These mature DCs process the respective antigens and display the epitope, the antigenic determinant of the antigens, on their cell surface via the major histocompatibility complex (MHC) molecules (Banchereau and Steinmann 1998). Antigen presentation by immature antigen-presenting cells (APCs) leads to antigen tolerance. Activated, mature DCs, on the other hand, also secrete cytokines, such as IL-1 β , IL-6 (Cumberbatch et al. 1996; Enk and Katz 1992), IL-8 and TNF α (reviewed by Kimber et al. 2011),

necessary for dendritic cell emigration, and express co-stimulatory molecules, such as CD86, CD54, CD40 and CD83 (Ozawa et al. 1996; Verrier et al. 1999), which are involved in T-cell interactions. Once activated, the DCs migrate to the local lymph nodes where they present the antigen to naïve T cells. If T cells recognize the allergen presented on mature DCs and the required secondary signals are present, they themselves become activated, proliferate and differentiate into T-effector and T-memory cells, which then circulate throughout the body. Upon second encounter with the same allergen, the APCs will present the allergen directly to preformed allergen-specific T cells, which ultimately leads to the inflammatory response typical for ACD (Banchereau and Steinmann 1998). After an individual has been sensitized to an allergen, further exposure with that particular substance can lead to the visible clinical symptoms typical for allergies. The extent of contact allergy is regulated by a balance between effector and inhibitory (T_{reg}) T cells present at the site of exposure to the skin sensitizer (reviewed by Peiser et al. 2012).

A number of factors determine whether a substance will function as contact sensitizer, including its ability to penetrate into the skin, the capacity to react with proteins present in the skin, as well as recognition of the chemical–protein complex by immune cells. Although the definition of haptens has been questioned and it has been suggested to classify substances according to the chemical reaction mechanism by which they can modify proteins instead (Aptula et al. 2007), for simplicity the terms hapten, pro-hapten and pre-hapten (as defined by Lepoittevin 2006) will be used throughout this article. While pro-haptens become metabolically activated through skin metabolism, pre-haptens are abiotically (e.g., oxidatively) activated in order to become haptens. These are then capable of binding to skin proteins, making these classes of sensitizers challenging for the development of non-animal assays, since metabolic activity may be limited.

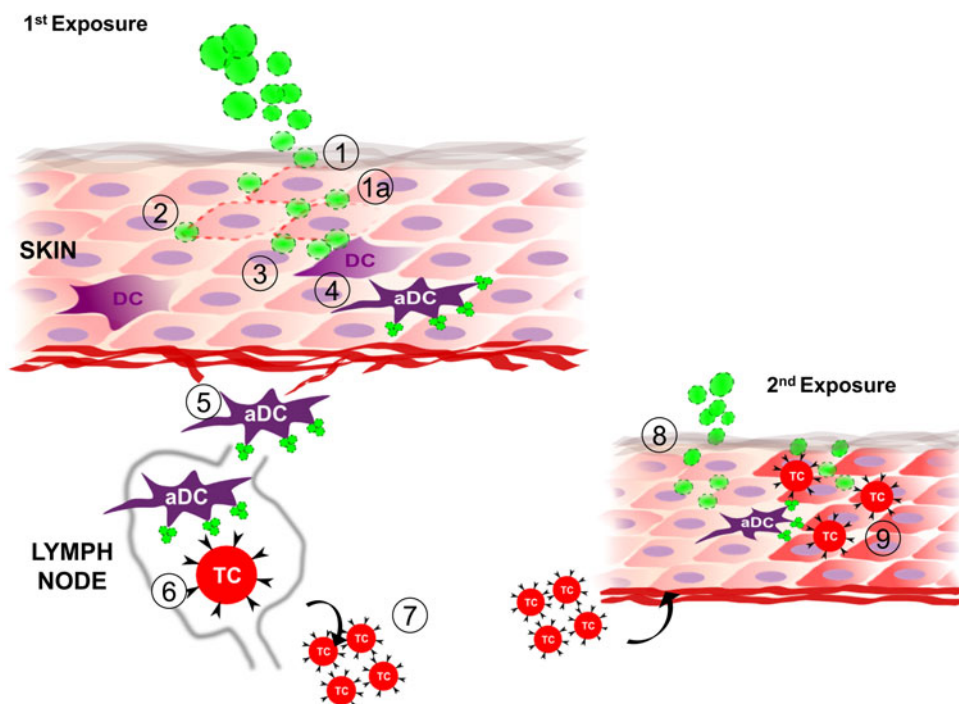
In vivo models

For reasons of comprehensiveness, a description of the *in vivo* methods used to obtain information on sensitization potentials shall precede the discussion of the current status of *in vitro* method development. These *in vivo* methods include guinea pig tests (GPT), the murine local lymph node assay (LLNA) and tests on human volunteers.

Guinea pig tests

The first guinea pig-based model, the occluded patch test of Buehler, was first described in a publication in (1965) and has since been used to identify possible skin sensitizers. A

Fig. 1 Schematic representation of the sensitization process according to the Adverse Outcome Pathway (AOP): haptens penetrate the epidermis (1). Haptens are either direct-acting electrophiles or are converted to electrophiles (1a), enabling covalent interaction with proteins (2) leading to the formation of complete antigens. Interaction with keratinocytes (KC, 3) and dendritic cells (DC) triggers DC activation and maturation (aDC, 4) and migration to the lymph nodes (5). There, the antigens are presented to naïve T cells (TC, 6). TC differentiate and proliferate (7). Upon a second contact with the hapten (8), TC are stimulated and mount a response leading to a dermal inflammation (9)



few years later, Magnusson and Kligman (1969) described the guinea pig maximization test (GPMT). Both guinea pig tests were adopted as OECD Test Guideline 406 in 1981 (OECD TG 406; revised in 1992). This guideline recommends using 10 (or 20) guinea pigs in the GPMT for the test substance-treated group and 5 (or 10) animals for the control groups, with the entire procedure taking 24 days to carry out. The Buehler test requires using a total of 30 guinea pigs and lasts up to 32 days. Besides the required use of adjuvant in the GPMT, both OECD-adopted protocols have the disadvantage that they rely on subjective endpoints. Performed in accordance with the standard procedure, the methods only provide a yes-or-no answer in regard to sensitization and thus have limitations when it comes to determining the potency of the tested substance. Apart from being time-consuming, the relatively large number of animals used and their limitations regarding potency, it has also been reported that the guinea pig tests can in rare cases result in both false-positive and false-negative prediction of the tested substances (Basketter and Kimber 2010). The advantage over the LLNA, which uses lymph node cell proliferation during the induction phase as a measure of sensitization, is that these tests cover both the induction and elicitation phases, the latter reflecting the actual allergy.

Murine local lymph node assay (LLNA)

Currently, the murine LLNA is the only formally validated method for evaluating the sensitizing potential of test substances. The LLNA has many advantages over the GPMT and the Buehler test. It is a method that takes the

3Rs into consideration by reducing animal distress and possibly animal numbers in comparison with the guinea pig tests. It provides a dose–response assessment, which can be used to assess the potency of the sensitizer, and it is less time-consuming than the GPT. In the LLNA, cellular proliferation in the draining lymph nodes is measured after repeated topical applications of test substances onto the ears of mice. The method was developed in the early 1990s (Kimber and Basketter 1992) and has been submitted to an extensive validation procedure. The OECD first adopted the LLNA in 2002 (OECD TG 429). According to this guideline, use of 20–25 mice is recommended covering one control group and three dose groups. A substance is classified as a sensitizer in the LLNA if a threefold or greater increase in cellular proliferation is achieved at one or more test concentrations as compared to the vehicle-treated control mice; the data must follow dose–response kinetics (Kimber et al. 2003). Since 2002, minor changes have been introduced into the assay protocol, resulting in a revision of the guideline in 2010 (OECD TG 429 2010).

Using the results of LLNA experiments based on the incorporation of radiolabeled thymidine as a proliferation marker, EC₃ values can be calculated. These values indicate the effective concentration (EC) required for a stimulation index (SI, that is, the ratio of the mean proliferation in each treated group to that in the concurrent vehicle-treated control group) of 3.0 in cells from draining lymph nodes. EC₃ values enable the categorization of substances into potency classes, with EC₃ values <0.1 % corresponding to extreme sensitizers, those ranging from ≥0.1 to <1 % to strong sensitizers, those from ≥1.0 to <10 % to

moderate sensitizers and EC3 values ≥ 10 % resulting in classification as weak sensitizers (ECETOC Report No. 46, 2008). Thus, in contrast to other available sensitization tests using animals, the EC3 values obtained from the LLNA can be used to assign the substances to a certain sensitization potency class (see also http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNA-pot/TMER.pdf).

Despite the advantages of the LLNA in comparison with the guinea pig assays, the LLNA also has drawbacks. The overall rate of false-positive results seems to be higher in the LLNA, and a number of classes of chemicals have been reported to lead to particularly high rates (e.g., surfactants; Ball et al. 2011; fatty acid-like substances, Kreiling et al. 2008; Basketter et al. 2008). Furthermore, strong irritants may cause false-positive responses in the assay (see OECD TG 429), possibly due to the fact that both irritants and sensitizers may induce LC migration and increased lymphocyte proliferation. However, in the case of sensitizers, the response is antigen specific, while irritants lead to an unspecific lymphocyte proliferation. During the formal validation process evaluated by ICCVAM, extensive LLNA data were compared to data from other animal methods and human data. The results revealed 72 % sensitivity, 67 % specificity and 72 % accuracy of the LLNA in relation to the human data (Haneke et al. 2001). Another study summarizing LLNA data also determined a 72 % accuracy of the LLNA compared to human data (reviewed by Anderson et al. 2011).

The standard LLNA protocol requires using radioactive thymidine (^3H -thymidine) for endpoint determination. However, a number of countries have limitations with respect to the use of ^3H -thymidine. This has forced the development of alternative, non-radioactive endpoint detection methods. Ehling et al. (2005) describe the inter-laboratory validation of a cell count method. Other alternatives to ^3H -thymidine endpoint detection have also led to satisfactory results: An interlaboratory validation study (Kojima et al. 2010) with seven participating laboratories compared the GPMT and the standard LLNA to the LLNA:BrdU-ELISA, a method using incorporation of BrdU into the DNA as a measure of proliferating cells. The LLNA:BrdU-ELISA showed a very good predictivity when compared to the GPMT (100 % specificity, sensitivity and accuracy), but lower specificity and accuracy when compared to the standard LLNA (75 % and 90 %, respectively; Kojima et al. 2010). A further LLNA variant uses ATP measurements as a measure of proliferation. These two methods have now been accepted on the level of the OECD (OECD TG 442 A and B). Recently, a comparison of the standard LLNA and an LLNA measuring lymph node cells counts (LNCC) was made in parallel with 22 performance standard substances. Of those 22 substances, 21 yielded the same results with respect to the classification of the

substances regardless of the method used (Basketter et al. 2011). A reduced LLNA (rLLNA; OECD TG 429) is a further 3Rs approach by limiting the dose groups to one dose instead of three. Animal numbers are thereby reduced, but information on potency is also lost. Therefore, this assay may be considered to distinguish sensitizers from non-sensitizers if dose–response information is not required.

Tests with human volunteers

Human sensitization data often originate from methods such as the human maximization test (HMT) described by Kligman (1966), diagnostic human patch tests for allergic responses (HPT), repeated open application tests (ROATs) or the human repeated insult patch test (hRIPT). However, for ethical reasons, sensitization testing on humans is difficult, methods are not always sufficiently standardized, and the readout is based on the same subjective endpoints as the GPT, that is, the visual assessment of skin reactions. Limited human data are therefore available for many substances. Furthermore, false-positive reactions also can occur, for example, to surfactant-based products, where irritation is sometimes incorrectly assessed as sensitization (Uter et al. 2005). In addition, a substance itself may not be a sensitizer, but due to cross-reactivities, allergic reactions may nevertheless take place. Therefore, human data must be evaluated with caution. These considerations underline the importance of defining a standardized data set for hazard and, most particularly, for potency assessments, which can then be used for the validation of alternative methods.

Cell types and cell culture conditions for in vitro assays

The choice of appropriate cell types is crucial for the development of in vitro models for the identification of potential sensitizers. The cell type used must mimic the in vivo function as closely as possible. Furthermore, the source of the respective cell type must be stable, reproducible and readily available (ideally not restricted by licensing fees) in order to be applicable for a practical test suitable for high-throughput testing. Examples for various cell lines as well as for primary cells that have been evaluated for their usefulness in predictive tests are presented below. In addition, a number of cell culture parameters are discussed, such as cell density, cell viability, culturing conditions and test protocols, which all are crucial for the outcome of the in vitro assays.

As discussed above, LCs and DCs are important dermal APCs playing a significant role in the development of ACD. These cells are characterized by their unique

morphology and their ability to take up and process antigens and to initiate subsequent immune responses (Banchereau and Steinmann 1998). Isolation of LCs from the human epidermis, which only constitute 1–3 % of all epidermal cells, has been shown to be difficult and to result in low yields (Hanau et al. 1988; Teunissen et al. 1988). Moreover, LCs may also spontaneously mature during preparation (Vandebriel and van Loveren 2010). To overcome these problems related to LCs, it has been strived to develop test protocols using dendritic-like cells or cell lines.

Cells derived from primary human sources have been used successfully to develop in vitro sensitization assays. These cells can be derived from human CD14+ peripheral blood monocytes (Lenz et al. 1993; Sallusto and Lanzavecchia 1994), from cord blood or from bone marrow-derived CD34+ hematopoietic precursor cells (Caux et al. 1997; Inaba et al. 1992). Especially, CD34+ DCs have proven valuable in in vitro sensitization test development (Reuter et al. 2011; Ade et al. 2009; Lambrechts et al. 2009, 2011; Schoeters et al. 2005, 2006, 2007; De Smedt et al. 2005; Verheyen et al. 2005). Nevertheless, these cells also have a number of drawbacks, including limited supply of cell sources, donor-to-donor variation in cell responsiveness and handling of primary human material (e.g., potentially infective material) as well as expensive and time-consuming culturing procedures. However, depending on the method and cell type used, a recent publication investigating the basal expression of CD86 on monocyte-derived DCs from almost 400 donors showed surprisingly low variability (Reuter et al. 2011). Therefore, depending on the method used, the impact of donor-to-donor variation might be less significant than originally perceived. The major advantage of using primary cells is that they are likely to retain almost full functionality and the metabolic capacities typical for the organ from which they were isolated, in this case the skin.

In order to overcome some of the disadvantages associated with DCs from primary sources, DC surrogate cell lines have been studied extensively. Advantages of such cell lines are that they can be cultured relatively easily; they are more amenable to standardization and have been shown to be capable to differentiate into a DC-like phenotype. In searching for DC surrogates suitable for in vitro assays, attention has been paid to sensitization-specific cell characteristics, such as sensitizer-induced up-regulation of cytokines (IL-1a, IL-1b, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18 and TNF α), altered expression of specific surface molecules (e.g., CD54, CD80, CD86 and MHCII; reviewed by Kimber et al. 2011) and, to a lesser extent, LC markers such as E-cadherin and Langerin (Dos Santos et al. 2009). The human myeloid cell lines THP-1 (Ade et al. 2009; Ashikaga et al. 2002; Sakaguchi et al. 2006; Yoshida et al.

2003), U937 (Ade et al. 2006; Python et al. 2007), KG-1 (Hulette et al. 2002) and MUTZ-3 (Azam et al. 2006; Johansson et al. 2011; Python et al. 2009) are all examples of extensively studied human myeloid cell lines that have been used successfully as DC surrogates in in vitro assays. Since choice of the most suitable cell line is assay dependent, it is essential to compare the performance of different cell lines: When Lambrechts and co-workers studied the use of THP-1 cells as possible surrogates for primary CD34+ DCs, gene expression analyses revealed that THP-1 monocytes correlated better with CD34+ DCs than THP-1 macrophages, but that CD34+ DCs still remained the better choice for the discrimination of (non)-sensitizing substances (Lambrechts et al. 2009).

Regardless of the origin of the cells (cell line or primary cells), every cell type has its own unique characteristics and capabilities, but also its limitations. Limited or differing metabolic capacities when compared to cells found in situ or even to primary cells is a characteristic that is generally observed in cell lines. For example, the dendritic surrogate cell line THP-1 is a cell type with incomplete metabolic activity. If this cell line is used in an in vitro assay, the system may not always be sufficient for the correct prediction of pro-haptens (Ashikaga et al. 2010). Other factors can also play a role. For instance, it has been suggested that the failure to produce mRNA encoding IL-8 in THP-1 cells after stimulation with pre- and pro-haptens might be due to chemical-induced destabilization of the mRNA. When measured at earlier time points, for example, 3 h instead of 24 h, pre- and pro-haptens induced elevated levels of IL-8 mRNA as well as p38 mitogen-activated protein kinase (MAPK) (Mitjans et al. 2010). It remains to be shown whether these results prevail when a larger panel of test substances is studied (Table 1).

In general, keratinocytes (KC), the dominating cells in the epidermis, are the first cell type to encounter substances that have penetrated through the stratum corneum of the skin. KCs are able to secrete various (pro-)inflammatory cytokines, chemokines and growth factors (Matsue et al. 1992), and they play an important role in immune-mediated diseases, including ACD (reviewed by Barker 1992; Corsini et al. 1995; Vandebriel et al. 2005). KCs have metabolic activities enabling conversion of inactive pro-haptens into biologically active haptens, and they express chemotactic factors and adhesion molecules upon exposure to sensitizers. In addition, KCs express Toll-like receptors (TLR), which are important proteins of the innate immune system. A range of cytokines and chemokines are produced upon TLR activation, which in turn attract other cells such as DCs (reviewed by van der Veen et al. 2011). They are thereby essential in recruiting immune cells to the exposed skin area, which can also enhance the immune responses leading to ACD. Furthermore, being relatively easy to

Table 1 An overview of cell-based in vitro and in chemico methods for the prediction of skin sensitizers

Model/assay	Cell line/cell type	Number of substances tested ⁺	Detection of pro-haptens	Potency category estimation	Stage of ECVAM evaluation	References
h-CLAT (CD54 & CD86 expression)	THP-1 cell line	>110 (>80 sens, >30 non-sens)	Partial	No	Almost completed	Ashikaga et al. (2002, 2006, 2010), Yoshida et al. (2003), Sakaguchi et al. (2006, 2010), Nukada et al. (2011a, b)
MUSST (CD86 expression)	U937 cell line	?	Partial	No	Almost completed	Ade et al. (2006), Python et al. (2007), Aeby et al. (2010)
NCTC2544/IL-18 intracellular	NCTC2544 cell line	18 (14 sens, 4 non-sens)	Yes	No	Submitted as full study, almost completed	Corsini et al. (2009), Galbiati et al. (2011), Teunis et al. (2012)
KeratinoSens [®]	HaCaT-derived cells (Luciferase reporter gene)	73 (49 sens, 24 non-sens)	Partial	No	Submitted	Natsch and Emter (2008), Natsch et al. (2009), Natsch (2010)
LuSens	HaCaT-derived cells (Luciferase reporter gene)	54 (35 sens, 20 non-sens)	Partial	No	Submitted	Bauch et al. (2012)
VITASENS [®] (gene expression assay)	CD34+DC cells	29 (17 sens, 11 non-sens)	Yes	Yes ^a		Hooyberghs et al. (2008), Lambrechts et al. (2010a, b, 2011)
GARD (gene expression assay)	MUTZ-3 cell line	40 (20 sens, 20 non-sens)	Yes	Yes		Johansson et al. (2011)
CeeTox (peptide binding & gene expression)	HaCaT cell line	97 (69 sens, 28 non-sens)	Yes	Yes		McKim et al. (2010)
DRPA	In chemico	88 (58 sens, 30 non-sens)	No ^a	Yes	Almost completed	Gerberick et al. (2004, 2007)
PPRA	In chemico enzyme activation	70 (45 sens, 25 non-sens)	Yes	Yes		Gerberick et al. (2009), Troutman et al. (2011)
LLNA	Mouse model	97 (67 sens; 30 non-sens vs. GPT)	Yes	Yes	Formally validated	Haneke et al. (2001)
EE	3D skin model	16 (16 sens)	Yes	Yes		Dos Santos et al. (2011)

The murine Local Lymph Node Assay (LLNA) is included for comparison

sens—sensitizer, non-sens—non-sensitizer

+ Numbers pertain to the cited references, ^ain combination with IC20 values

culture, KCs represent an important cell type suitable for the development of *in vitro* skin sensitization assays. They have been used in a number of methods such as cell assays measuring intracellular IL-18 (Corsini et al. 2009; Galbiati et al. 2011; Teunis et al. 2012) and in the KeratinoSensTM assay (Emter et al. 2010; Natsch and Emter 2008; Natsch et al. 2010, 2011) described in the following sections of this article.

As a rule, the metabolic status of the cell type to be used in a test method or testing strategy must be taken into account. Choice of an appropriate cell type serves to reduce deficiencies in metabolic activities, although a single-cell system is unlikely to represent the full range of metabolic activities available *in vivo*. KCs can play an important role in improving the metabolic activity of *in vitro* systems, since they are known to participate in the haptenization process also by supplying the enzymes necessary for the metabolic activation of pro-haptens (Kim et al. 2009).

In order to develop a method suitable for the detection of pro-haptens, a co-culture system comprising metabolically competent KCs (HaCaT) and dendritic cell surrogates (THP-1) has been developed. Although a limited panel of substances was tested, the co-cultures showed a better potential to correctly classify pro-haptens as compared to mono-cell cultures possibly due to cell signaling pathways triggered by crosstalk between the different cell types (Hennen et al. 2011). A similar study design used THP-1 cells co-cultured with KCs and demonstrated augmentation of CD54 and CD86 expression after stimulation with 6 sensitizers including the pro-hapten isoeugenol (Cao et al. 2011). A method termed loose-fit co-culture-based sensitization assay (LCSA) comprising co-cultures of KCs and dendritic-related cells enabled correct classification of the pro-hapten isoeugenol (Schreiner et al. 2008). This assay might also be able to discriminate between sensitizing and irritating properties of compounds (Sonnenburg et al. 2012).

Cell culture conditions for *in vitro* assays

Selecting appropriate concentrations of the test substances when stimulating cells *in vitro* is a crucial parameter for the elicitation of an immune response. As a general rule, cytotoxicity assays are used to establish suitable test concentrations for the subsequent sensitization tests. Viability of the cells at different test substance concentrations is assessed, and the concentrations resulting in viability above a certain threshold are used for the stimulation experiments. Since toxic effects may introduce artefacts, such as false-positive or negative responses, the maximum tolerated cytotoxicity is a critical parameter when developing *in vitro* sensitization tests. These viability thresholds require adaptation to the cell lines and test methods used. For

instance, the maximum tolerated cytotoxicity was set at 25 % relative cytotoxicity in the h-CLAT assay employing THP-1 cells (Sakaguchi et al. 2010), at 20 % for both the MUTZ-3 migration assay (Gibbs et al. 2012) and a gene expression assay utilizing CD34+ DCs (Hooyberghs et al. 2008), at 15 % in a gene expression assay employing DCs derived from peripheral blood mononuclear cells (Gildea et al. 2006), at 30 % in different assays using U937 cells (Python et al. 2007; Aeby et al. 2010) and even as low as 10 % relative cytotoxicity in gene expression assays based on MUTZ-3 cells (Johansson et al. 2011; Python et al. 2009).

Sakaguchi et al. (2009) reported that most allergens were predicted to be sensitizers within the range of 65 % to almost 90 % cell viability when using the h-CLAT assay with THP-1 cells. The authors assume that a certain degree of cytotoxicity is necessary to enhance sensitization responses and to clearly demonstrate sensitization effects in DCs or LCs (Hulette et al. 2005; Jacobs et al. 2006). Nukada et al. (2011a) reported that both allergens and non-allergens are able to induce cytotoxicity. Even though the proportion of early apoptotic cells was significantly higher after stimulation with four different contact allergens when compared to non-allergens, this effect could also be due to other substance-specific properties. Notably, the induction pathway of apoptosis did not affect the expression of cell surface CD54 and CD86 in THP-1 cells, suggesting that allergen-induced up-regulation of the surface markers and apoptosis are regulated by independent pathways (Nukada et al. 2011a). In a keratinocyte-based assay measuring intracellular IL-18, it was demonstrated that cytotoxicity is not a prerequisite to elicit sensitization and that significant and sensitizer-specific increase in IL-18 occurs at concentrations above the cell viability of 80 % (CV80 value; Corsini et al. 2009; Galbiati et al. 2011).

It is increasingly being recognized that the determination of the appropriate number of cell passages is a further important parameter to be taken into account when standardizing cell culture assays, since maintenance of the cell line has important implications for the performance of the respective test method. Cells originating from older passages of THP-1 cells showed lower allergen responses than earlier passages. Therefore, THP-1 maintenance should be limited to less than 2 months in culture from the original stock, and these cells should be used for a maximum of 10 passages (An et al. 2009). Likewise, the U937 cell line should be passaged for a maximum of 4 weeks (Python et al. 2007). Regarding the MUTZ-3 cell line, it has been suggested to use the cells between passage no. 6 and 40 after thawing in order to achieve optimal results in a migration assay (Gibbs et al. 2012) and not to grow them for more than 20 passages or two months after thawing for best performance in a gene expression assay (Johansson et al. 2011).

Skin models

In vitro three-dimensional epidermal equivalents (EE) consist of KCs cultured on a filter or matrix where a fully differentiated epidermis with stratum corneum develops at the air–liquid interface. Since cells grow in multilayers in EE and junctions are formed between the cells, these models more closely resemble in vivo skin than cell culture models. Epidermal models are believed to be useful for sensitization testing, since they represent the location of the very first phase of skin sensitization where the substance penetrates the stratum corneum and reaches viable keratinocytes within the epidermis (Dos Santos et al. 2011). Several skin equivalents are commercially available, such as EpiDerm™ (MatTek Corporation), EST1000™ (Epidermal Skin Test-1000, CellSystems), RHE™ (Reconstructed Human Epidermis) and EpiSkin™ (both SkinEthic Laboratories). Assays using SkinEthic™, EpiDerm™ and EpiSkin™ have been validated for skin irritation testing and have been adopted by the OECD for skin irritation and/or corrosion testing (OECD TG 439) as well as for skin penetration assessment (OECD TG 428; Schreiber et al. 2005; Schäfer-Korting et al. 2008). Further developments include full-thickness skin models, which incorporate both the epidermis and the dermis. In these models, crosstalk between the KCs and the fibroblasts allows the development of tissues, which more closely mimic the human skin (Mewes et al. 2007; Ackermann et al. 2010). Although three-dimensional models offer several advantages over monolayer cultures, those commercially available still lack the presence of immune cells, which, however, are essential for the sensitization process in vivo. Attempts are being made to incorporate antigen-presenting cells into 3D skin models, including those that are commercially available. Facy et al. (2005) incorporated CD34+–derived LCs from cord blood into the EpiSkin™ model and confirmed increased IL-1 β and CD86 expression after allergen exposure. Furthermore, in a model comprising KCs, fibroblasts and DCs, an increased expression of CD86 was found after stimulation with the strong sensitizer DNCB (Uchino et al. 2009). Other researchers created a skin model comprised of dermal macrophages introduced into a rigid scaffold of dermal fibroblasts, a model considered valuable for dermatological research (Bechetoille et al. 2011).

In vitro cell-based assays

In vitro tests for use as alternatives to animal testing must mimic the very complex interactions between substances and the different parts of the immune system. As depicted above, in the in vivo situation, the substance must first

penetrate the skin and thereafter react with endogenous proteins present in the skin. Next, the hapten–protein complexes are internalized and processed by immature DCs, which in turn become activated. The activated and mature DCs migrate to draining lymph nodes and present the processed hapten–protein complex to T cells, which orchestrate the specific immune response (Banchereau and Steinmann 1998). Assay sensitivity and specificity are important parameters to be taken into consideration when evaluating the suitability of in vitro cell-based assays to mirror these complex mechanisms. For many industry sectors, predicting a non-sensitizer as a sensitizer (i.e., a false positive) is more acceptable than vice versa, that is, to predict a sensitizer as a non-sensitizer (a false-negative result) (Chaudhry et al. 2010), provided that false predictions are not excessive. Furthermore, for proper risk management, the quantitative risk must be determined so that information on allergen potency is required, similar to the EC3 values obtained from LLNA studies.

When evaluating whether alternative in vitro methods enable correctly classifying the substances tested, in vitro data are frequently compared to LLNA data. However, the LLNA, as well as the GPTs, is judged to be less than 75 % accurate when compared to human data (Dean et al. 2001; reviewed by Anderson et al. 2011; SCCNFP/0120/99). Thus, if the in vitro method contains additional inaccuracies, the errors are likely to multiply when an in vitro method is assessed against a single in vivo method (Basketter and Kimber 2010). The current replacement rationale is that data from different non-animal test methods will have to be combined in order to get useful skin sensitizer potency information (Dos Santos et al. 2009; Jowsey et al. 2006; Maxwell et al. 2011). The following sections summarize and discuss the current state of development and evaluation of different in vitro assays for the predictive identification of skin sensitizers.

CD86 induction assays using U937 cells

As regards phenotypic changes induced by contact sensitizers in dendritic cells and myeloid cell lines, to date, CD86 is the most extensively studied surface marker (e.g., Ade et al. 2006; Ashikaga et al. 2002; Ashikaga et al. 2010; Azam et al. 2006; Johansson et al. 2011; Python et al. 2007; Yoshida et al. 2003). A dendritic-like cell line activation test using U937 cells was developed by researchers at Procter and Gamble (Python et al. 2007). This assay utilizes the fact that CD86 expression is up-regulated on the surface of U937 cells after stimulation with contact sensitizers (Python et al. 2007). A further U937-based cell activation test, termed MUSST (myeloid U937 skin sensitization test), was developed by L'Oréal. In assessing the accuracy of this assay using 99 substances, the concordance with human

clinical data was found to be 85 % (reviewed in Aebly et al. 2010; Maxwell et al. 2011; Thyssen et al. 2007). Two subsequent ring trials conducted by Cosmetics Europe/COLIPA determined interlaboratory reproducibility to be robust (reviewed by Maxwell et al. 2011). In a recently studied U937 cell line activation test (Bauch et al. 2011, 2012), termed mMUSST, U937 cells cultured on 96-well plates are stimulated with different concentrations of test substances for 48 h. Subsequently, the surface expression of CD86 is measured with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody and analyzed by flow cytometry. A substance is considered to be a sensitizer if it induces a concentration-dependent increase in CD86 expression at non-cytotoxic concentrations in two independent experiments. The mMUSST was evaluated with 54 substances revealing a sensitivity of 75 % and a specificity of 100 % when compared to human data (Bauch et al. 2012). A study employing MUTZ-3 cells (instead of U937 cells) investigating 20 sensitizers and 20 non-sensitizers showed that using CD86 as a single marker resulted in a sensitivity of only 50 %, but a specificity of 100 % (Johansson et al. 2011). Currently, the MUSST assay is being subjected to the pre-validation process at ECVAM.

Human cell line activation test (h-CLAT)

During the induction phase of skin sensitization, the co-stimulatory molecules CD54 and CD86 expressed on the surface of dermal LCs play important roles in the antigen-presenting process. Initial studies using THP-1 cells as DC surrogates revealed elevated CD86 and CD54 expression after stimulation with contact sensitizers (Ashikaga et al. 2002; Yoshida et al. 2003). These findings paved the way for the development of the human cell line activation test (h-CLAT) by researchers at Kao and Shiseido. The h-CLAT is a thoroughly studied method, which furthermore has almost completed (pre)-validation under the auspices of ECVAM. In the h-CLAT assay, THP-1 cells cultured in 24-well plates are stimulated with test substances. Thereafter, CD54 and CD86 molecules are detected by specific antibodies (after blocking of Fc receptors (FcR) present on THP-1 cells) and analyzed by flow cytometry. Substances are regarded as sensitizers if the increase in relative fluorescence intensity (RFI) is ≥ 150 % for CD86 and ≥ 200 % for CD54 (the latter prediction model having been modified from 150 to 200 % in 2010) compared to the vehicle control. In addition, 2 of 3 independent data at any dose should exceed the positive criteria (Sakaguchi et al. 2006, 2010).

Extensive work has been performed to optimize the experimental conditions of the h-CLAT (Ashikaga et al. 2006; Sakaguchi et al. 2006) and to investigate the relationship between CD54/CD86 expression and THP-1 cell

viability (Nukada et al. 2011a; Sakaguchi et al. 2009). Furthermore, interlaboratory studies were conducted (Nukada et al. 2011b; Sakaguchi et al. 2006, 2010) to test the performance of the method. To investigate protocol transferability, interlaboratory reproducibility and predictive capacity, Cosmetics Europe/COLIPA coordinated ring trials during which the h-CLAT method was evaluated in five independent laboratories in Japan, Europe and the United States. In comparison with the LLNA, 84 % of the substances were correctly identified (Sakaguchi et al. 2010). A further study comparing the h-CLAT to the LLNA, performed using 100 test substances, also revealed an 84 % correlation between the h-CLAT and the LLNA. Nine substances were false negative in the h-CLAT. In general, the method was more reliable in identifying extreme and strong sensitizers, whereas weak sensitizers and non-sensitizers resulted in lower detection rates (Ashikaga et al. 2010). Another investigation of the predictive performance of the h-CLAT was performed with 66 substances whereof 51 represented sensitizers. In this study, 88 % of the allergens were correctly identified, and the corresponding figure for non-allergens was 67 %; the overall agreement between h-CLAT data and LLNA data was 83 %. Human data were available for 38 of the substances; in that comparison, the overall accuracy was 79 %. The study also showed that extreme, strong and moderate sensitizers had the highest predictive performance, while the sensitivity was lower for weak sensitizers (Nukada et al. 2011b). Furthermore, the h-CLAT method has limitations when it comes to identifying pre- and pro-haptens, which can give rise to false-negative results (Ashikaga et al. 2010). Further possible reasons why false-negative results in h-CLAT experiments, as well as any other cell-based in vitro method, can occur are low water solubility of the test substances under the conditions used in the assay, insufficient metabolic activity of the cell line for pro-haptens or insufficient air oxidation of pre-haptens.

Assays using additional biomarkers and approaches

Besides the extensively studied cell surface molecules (e.g., CD80, CD86 and CD54) evaluated for their usefulness in skin sensitization tests, several other biomarkers have been investigated at the protein level. For example, CD40 has been showed to be a useful complementary marker to CD54 and CD86 in THP-1 cells (An et al. 2009; Miyazawa et al. 2007). In THP-1 cells, the expression of macrophage inflammatory protein-1 β (MIP-1 β) was shown to be increased upon stimulation with sensitizers, but not with non-sensitizers (Lim et al. 2008), and IL-1 β was demonstrated to be up-regulated by sensitizers (Python et al. 2007). Furthermore, TNF α has been described to be involved in skin sensitization (Miyazawa et al. 2007,

2008), and neutralization of TNF α activity was shown to suppress IL-8 production in allergen-stimulated THP-1 cells (Nukada et al. 2008).

Mitogen-activated protein kinases (MAPKs) are enzymes that respond to extracellular stimuli and regulate a number of cellular events such as gene expression, mitosis, cell differentiation and cell survival. In mammals, three different types of MAPK signaling pathways exist, namely extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPK. Several studies have shown induction of different pathways depending on the substances tested (Aiba et al. 2003; Becker et al. 2003; Koeper et al. 2007; Trompezinski et al. 2008). Therefore, it is likely that cell signaling can be used to identify sensitizers. Furthermore, Aiba et al. (2003) showed that the irritant sodium dodecyl sulfate (SDS), which oftentimes causes false-positive responses in *in vitro* assays (Basketter and Kimber 2010), does not activate any of the three MAPK pathways.

A number of researchers have focused on IL-8 expression in *in vitro* stimulated cells, and IL-8 secretion from sensitizer-stimulated THP-1 cells has been widely studied (Mitjans et al. 2008; Miyazawa et al. 2007; Nukada et al. 2008; Sakaguchi et al. 2006). Apart from the allergen-specific up-regulation of IL-8, the study by Miyazawa and co-workers showed that all allergens led to p38 MAPK activation, suggesting that p38 MAPK represents a common pathway triggered by allergens. Furthermore, specific inhibition of the p38 MAPK pathway has been shown to suppress IL-8 production in allergen-stimulated THP-1 cells (Nukada et al. 2008). In the same study, a sensitivity of 81 % and a specificity of 58 % were achieved with the 16 allergens and 7 non-allergens tested. On the mRNA level, sensitizer-induced up-regulation of IL-8 has also been described using a stable THP-1-derived IL-8 reporter cell line (THP-G8) harboring a luciferase gene under control of the IL-8 promoter (Takahashi et al. 2011). Besides the examples given for THP-1 cells, elevated levels of IL-8 have also been found in sensitizer-stimulated U937 cells (Python et al. 2007) and monocyte-derived DCs (Toebak et al. 2006).

KC-based assays

KCs are the dominant cells in the epidermis, and they are known to play a key role in the activation of LCs in response to stimulation by pathogens or chemicals. Attention has been paid to employing KCs in *in vitro* assays for the evaluation of potential sensitizers. Furthermore, sensitizer-induced signaling pathways have also been utilized in skin sensitization prediction assay development. In this context, different research groups depicted the importance of the Nrf2-Keap1 pathway in skin

sensitization (Ade et al. 2009; Natsch 2010; Natsch and Emter 2008; Vandebriel et al. 2010): Under physiological conditions, the transcription factor Nrf2 forms a complex with the sensory protein Keap1. In response to oxidative stress, the highly reactive cysteine residues of Keap1 are activated and Nrf2 is released. Free Nrf2 then translocates into the nucleus, where it forms a complex with other molecules. This complex then binds to the so-called anti-oxidant response element (ARE) in the promoter region of several genes, including HMOX1 and NQO1. Skin sensitizers, which are known to be reactive to cysteine, are thought to be able to bind the cysteine residues of Keap1, thereby enhancing Nrf2 release (Emter et al. 2010; Motohashi and Yamamoto 2004; Natsch 2010). These findings led to the development of the KeratinoSensTM assay by researchers at Givaudan. The KeratinoSensTM assay employs a cell line derived from HaCaT cells, which carries a reporter gene for luciferase under control of the ARE response element and monitors Nrf2 transcription activity. The principle of the assay is that exposure to skin sensitizers leads to activation of the Nrf2 pathway and hence luciferase expression (Natsch and Emter 2008; Natsch 2010). To date, more than 100 substances have been assessed (e.g., Natsch et al. 2009). When the assay was subjected to intra- and interlaboratory testing with 5 participating laboratories, an accuracy of 85 % was achieved using 28 blinded test substances. Furthermore, the assay was easily transferable and reproducible between the laboratories (Natsch et al. 2011). The results achieved with the KeratinoSensTM assay are encouraging, and the assay is currently under (pre)-validation in collaboration with EC-VAM and approaching the stage of final decision. Along with the direct peptide reactivity assay (DPRA, see below) and possibly the h-CLAT, this method may well be one of the first to gain regulatory acceptance.

A limitation of the current KC-based *in vitro* assays is that they only result in yes-or-no answers and do not provide potency data. In order to overcome this problem, an intracellular IL-18/NCTC2544 assay was used in a two-tiered approach together with an EE assay (Teunis et al. 2012). The EE potency assay on its own is not able to distinguish sensitizers from irritants, but it does allow estimation of potency once the differentiation has been made. It was suggested that the intracellular IL-18/NCTC2544 assay could be used in a multistep approach in which this assay is used in the first tier to determine whether a substance is a sensitizer or not. If the substance is classified as a sensitizer, the EE assay is performed in the second tier to quantify the potency of the sensitizer. Teunis et al. (2012) underline that skin sensitization is a complex process and that it might be necessary to combine this two-tiered approach with further methods investigating additional markers.

Still in a very early stage of development, a further approach utilized fluorescent thiol-reactive sensitizers incubated with KCs. The haptens were rapidly internalized, and the KCs released membrane blebs containing haptened keratins 5 and 14. Although extensive further work remains to be accomplished, this assay might serve to estimate allergen potency, since the number of cells forming membrane blebs increased with the increased potency of the contact allergens tested (Bauer et al. 2011).

Assays using sensitizer-specific T-cell responses

The activation and clonal expansion of antigen-specific effector, memory and regulatory T cells are thought to be central to the skin sensitization process (Jowsey et al. 2006; Maxwell and MacKay 2008). It is reasonable to assume that sensitizer-induced T-cell responses can be used as a mechanistic basis for in vitro assays. Despite efforts during the last years, a test method for generating data on sensitizer-induced T-cell responses remains a gap to be filled. Unfortunately, there are a number of challenges to be overcome before a T-cell-based assay is available, such as the fact that any pool of naïve T cells of a particular specificity only represents a very tiny fraction of 1 % thereof. Although some contact allergens have larger T-cell repertoires, the measurement of allergen-specific T-cell responses in vitro is still technically challenging (Kimber et al. 2011). However, there are protocols describing the proliferative responses of naïve T cells after stimulation with sensitizers (Dai and Streilein 1998; Dietz et al. 2010; Guironnet et al. 2000; Kratseva et al. 1996; Moulon et al. 1993; Rougier et al. 2000; Rustemeyer et al. 1999; with a review by Martin et al. 2010). Although inclusion of a T-cell-based assay in an integrated test strategy appears scientifically sound due to the importance of the T cells in ACD, it is more likely that other cell-based assays will be selected, at least for the time being, since the development of the T-cell-based assays are not yet far advanced.

Assays using reconstructed tissue models

A recent study described an assay comprising in vitro reconstructed human EE to determine sensitizer potency (Dos Santos et al. 2010). The EE-EC₅₀ (effective concentration that results in a 50 % decrease in cell viability) and the EE-IL-1 α _{10 $\times$} values (concentration that increases IL-1 α secretion by 10-fold) were assessed. Of the 16 skin sensitizers evaluated, 12 skin sensitizers yielded values for both endpoints. The EE-EC₅₀ and EE-IL-1 α _{10 $\times$} values of eight sensitizers correlated well with the sensitization potency observed in the LLNA or with human data. Additionally, correct potency classification was obtained when using two commercially available EE models;

however, IL-1 α release was lower (twofold), and only two sensitizers were tested (Dos Santos et al. 2011). The authors attributed the correlation of IL-1 α with potency partially to the role of irritation in sensitization potency. Since the EE assay does not distinguish between sensitizers and non-sensitizers, it is considered useful in a two-tiered approach to determine the sensitizing potency of a substance that has already been classified as a sensitizer in tier 1 (Dos Santos et al. 2011). The EE assay is currently being evaluated with 13 sensitizers in an interlaboratory study in five European laboratories (Teunis et al. 2012).

An example of an attempt to assess the sensitization potency of chemicals is the SENS-IS[®] assay, an EpiSkin[®]-based model where specific biomarkers are analyzed quantitatively by RT-PCR. Initially, the gene markers were selected after analyzing mouse skin challenged with sensitizers, and the relevance of the mouse data was confirmed by analysis of the markers in blisters from human skin exposed to two sensitizers (nickel and fragrance mix). The SENS-IS[®] assay was tested with 29 different substances. Genes were grouped into 1) irritant genes, 2) ARE genes and 3) genes specifically induced by sensitizers, but not belonging to the ARE group (SENS-IS[®] genes). Sensitizing potency is reflected by the number of over-expressed genes in groups 2) and 3) and not by the x-fold change per gene. The method is claimed to have good specificity, sensitivity and concordance to LLNA (Martinozzi-Teissier et al. 2011, with additional information to be found at: http://www.skinethic.com/iso_album/evaluation_of_sens-is.pdf). In parallel, the same research group developed the IRR-IS[®] assay, an in vitro test for skin irritation quantification based on EpiSkin[®] stimulation and gene expression analysis by RT-PCR (Cottrez et al. 2011).

Migration assays

Another parameter worth taking into consideration when developing in vitro assays is the impact of chemical allergens on the mobilization and migration of DC-like cells (Dos Santos et al. 2009). A transwell assay has been described based on the selective migration of the DC-like MUTZ-3 cells toward the chemokine CXCL12 following exposure to skin sensitizers (Ouweland et al. 2010). The method was tested in an interlaboratory study with 3 participating laboratories during which all 5 substances were correctly identified. In addition, the protocol was transferable and the variability between the laboratories was low (Rees et al. 2011). However, the panel of substances tested was very limited (3 sensitizers and 2 non-sensitizers). Furthermore, this method is extremely complex and resource intensive, and its robustness has not yet been systematically assessed. Based on the same principle, another paper described the sensitizer-specific migration of

MUTZ-3 cells toward CXCL12 and, on the other hand, non-sensitizer-induced migration toward CXCL5. The protocol was tested in three European laboratories with 7 sensitizers and 2 non-sensitizers, all of which were correctly identified. Furthermore, the assay was easily transferable, and the results were consistent between the laboratories (Rees et al., 2011). Although these results appear promising, the number of substances tested in migration assays is still very limited. Furthermore, these assays are very complex and therefore not amenable to routine testing. Thus, the usefulness of this kind of tests remains to be elucidated.

Gene expression analysis

It has been suggested that the response patterns of biomarkers at the protein level (i.e., protein expressed on the cell surface and/or secreted cytokines and mediators) might have a limited dynamic range and might be substance dependent (Casati et al. 2005). This observation has encouraged the development of transcript profiling tests. During the last decade, several publications describing sensitizer-induced gene expression patterns have emerged. Using different cells types and cell lines, the expression of different genes has been analyzed using microarrays and/or quantitative/real-time PCR. Using peripheral blood mononuclear cells (PBMCs) as a source for DCs, Gildea et al. (2006) identified 10 genes that were selectively up-regulated after stimulation with 11 skin sensitizing substances of different potencies. However, employing high numbers of gene markers in combination with few test substances *per se* increases the likelihood of correct predictions. The authors speculate that in the future information on the expression of the genes Notch3, AKR1C2, ABCA6 and SLAM may contribute to the classification of allergens into potency categories.

Using microarrays followed by quantitative PCR analysis, Python et al. (2009) determined gene expression in DCs derived from PBMCs and in MUTZ-3 cells. Ten genes relating to immune and/or inflammatory responses (including CD86, IL-1 β and IL-8) were significantly up- or down-regulated in both PBMC-DCs and MUTZ-3 cells. The authors concluded that multiple markers will be required to enable correct detection of putative sensitizers. The genes PIR, TRIM16, CES1 and NQO1 were modulated by most of the sensitizers included in the study. Gene expression patterns in THP-1 cells have also been studied, and 13 candidate genes were investigated by real-time PCR after stimulation with test substances. The expression of the genes IL-8, HMOX1 and PAIMP1 enabled the prediction of contact sensitizers with a sensitivity of 74 % (Arkusz et al. 2010).

Extensive studies on gene expression in CD34 + progenitor-derived dendritic cells from cord blood

CD34 + progenitor-derived DC [CD34-DCs] have been performed by researchers at VITO/CARDAM. A classification model named VITOLENS[®] has been developed, which enables discriminating sensitizing from non-sensitizing substances, based on gene expression analysis of 13 genes in CD34-DCs. In total, less than 30 substances have been tested (Hooyberghs et al. 2008; Lambrechts et al. 2009, 2010a, b, 2011; Schoeters et al. 2005, 2006, 2007; Verheyen et al. 2005), demonstrating a sensitivity of approximately 80 % (Hooyberghs et al. 2008). Again, since the number of investigated genes is high, it remains to be established whether the results can be confirmed if an extended panel of substances is tested in combination with a reduced number of gene markers. However, also when investigating the genes CREM and CCR2 only, a sensitizer-specific response was observed, even though there was a high degree of variability in the responses (Lambrechts et al. 2011). Further attempts have aimed at developing in vitro models that enable predicting the sensitizing potency of substances (comparable to the EC3 values in the LLNA). CD34-DCs were exposed to 15 substances with known EC3 values at substance concentrations that yielded 20 % cell damage (IC20), and the gene expression of CREM and CCR2 was investigated. Linear regression with IC20 values and expression changes in CCR2 and CREM was performed and compared to the EC3 values in the LLNA. For 13 of the 15 substances tested, a linear correlation was established between the VITOLENS[®] assay and LLNA EC3 values (Lambrechts et al. 2010b).

Contact sensitizers are believed to have an intrinsic chemical reactivity leading to cellular stress, and the Keap1/Nrf2 signaling pathway is known to play a major role in the occurrence of cellular stress (Han et al. 2007; Lewis et al. 2006; Ryan et al. 2004). The role of the Keap1/Nrf2 pathway in THP-1 and CD34-DCs has been studied using real-time PCR analysis of the genes as well as Western blot analysis of accumulated Nrf2 protein. Sensitizers, but not irritants, were shown to cause an increased expression of the genes HMOX1 and NQO1 as well as accumulation of Nrf2 protein (Ade et al. 2009). Using different cell types, other authors have also referred to HMOX1 (Ade et al. 2009; Arkusz et al. 2010; Johansson et al. 2011; McKim et al. 2010) and NQO1 (Ade et al. 2009; Johansson et al. 2011; McKim et al. 2010) as genes up-regulated after stimulation with sensitizers.

Using the Affymetrix GeneChip[™], Johansson et al. (2011) analyzed 200 biologically relevant transcripts from MUTZ-3 cells stimulated with 20 sensitizers and 20 non-sensitizers. Although all substances were correctly identified, the potency prediction did not fully match corresponding LLNA data. These findings support the assumption that the current sensitizing potency categorization may need revision. The authors state that the number

of genes can be reduced to 11 without impairment of the correct classification of the test substances (Johansson et al. 2011).

Despite ongoing extensive research on novel biomarkers at the transcriptional level, to the best of our knowledge no assay has yet entered the stage of (pre)validation at ECVAM or ICCVAM, or any other institution. A reason for this could be that it is difficult to reproduce gene expression changes and to completely standardize the RT-PCR measurements. In addition, the number of substances tested in the above-mentioned assays is still very limited. And importantly, due to the complexity of these methods, the number of gene markers to be included in any assay must be limited in order to obtain a practical and cost-effective test.

In chemico methods

The correlation between skin protein reactivity and skin sensitization potential has been known for many decades (Dupuis and Benezra 1982; Landsteiner and Jacobs 1936; Lepoittevin et al. 1998). It is widely accepted that if a substance is capable of reacting with proteins in the skin, the substance, either directly or after biotransformation, has the potential to act as a skin allergen (although by far not every substance is then a sensitizer). In recent years, advances have been made in the field of in chemico methods. It is not unlikely that such methods will be included in future test batteries for the evaluation of potential skin sensitizers.

The direct peptide reactivity assay DPRA

The direct peptide reactivity assay (DPRA) is an in chemico method for predicting the sensitization potential of chemicals. Developed by Gerberick and co-workers in 2004, it is a cell-free assay in which test substances are incubated with synthetic model peptides and thereafter analyzed by high-performance liquid chromatography (HPLC). The test is based on the fact that the majority of chemical allergens are electrophilic and therefore capable of reacting with nucleophilic amino acids such as cysteine or lysine (Gerberick et al. 2004). To investigate the predictive performance of DPRA, 82 chemicals were tested for their ability to react with reduced glutathione or with two synthetic peptides containing either a single cysteine or lysine, resulting in a prediction accuracy of 89 % compared to LLNA data. In general, moderate to extreme allergens resulted in high peptide depletion, while weak sensitizers and non-sensitizers demonstrated significantly less peptide depletion. Gerberick et al. (2007) suggested that the DPRA could be used as a first-tier screening method to determine

skin sensitizers. The prediction model developed for DPRA uses a classification tree approach to assign different reactivity categories to the peptide depletion data. In this approach, 89 % concordance with LLNA potency categories was found following two rounds of ring trials (reviewed by Maxwell et al. 2011). In a further evaluation of the DPRA (Bauch et al. 2011), analysis of 23 substances resulted in a sensitivity of 94 % and a specificity of 86 % with α -hexyl cinnamic aldehyde incorrectly predicted as negative and SDS as a false positive. The DPRA has almost completed (pre)-validation under the auspices of ECVAM.

Further developments of peptide reactivity assays

Peptide depletion might not always be sufficient to classify a substance as a sensitizer or non-sensitizer. Natsch and Gfeller (2008) describe a modification of the original DPRA. Using a combination of LC–MS analysis and detection of free thiol groups, this approach allowed simultaneously determining peptide depletion, peptide oxidation (dimerization), adduct formation and thiol reactivity. The assay was evaluated using 80 chemicals. While adduct formation was only observed for sensitizers, peptide depletion due to oxidation reaction was also observed for two non-sensitizers, suggesting that peptide depletion alone is not a sufficient evidence for rating a substance as a sensitizer (Natsch and Gfeller (2008). Furthermore, the original DPRA protocol is unable to correctly identify pro-hapten chemical sensitizers, since they require activation in order to become reactive. It has been estimated that one-third of the known skin sensitizers require some kind of activation to become protein reactive and hence capable of acting as skin sensitizers (Smith and Hotchkiss 2001). To overcome the lack of enzymatic activity, the peroxidase peptide reactivity assay (PPRA) was developed where horseradish peroxidase and hydrogen peroxide were introduced to mediate an activation step. Using this approach, significantly increased peptide depletion was detected following co-incubation with the enzymes (Gerberick et al. 2009). A further improvement to the method was achieved by incorporating lysine into the PPRA, and an analysis of 70 test compounds led to promising results (Troutmann et al. 2011). Natsch et al. (2007) found low sensitivity (50 %) for weak allergens using a slightly modified version of the original assay by Gerberick et al. (2004). Assay sensitivity in regard to weak allergens could be increased by the inclusion of physiologically reactive peptides. In order to maximize the information that can be obtained from peptide reactivity assays, Aleksic et al. (2009) extended the standard approach to cover six single nucleophilic peptides and analyzed the outcome using liquid chromatography tandem mass spectrometry (LC–MS/MS). Thereby, both quantitative (percent depletion) and qualitative (number of adducts

formed and peptide dimerization for Cys-containing peptide) data could be obtained.

Other peptide binding methods

The PEPT-IS[®] assay utilizes binding to lipocalin to assess the potential allergenicity of chemicals. The peptide was selected from a structurally conserved region between human lipocalins and the allergen epitope of Bos d 2 (allergenic protein from cattle). After investigation of 30 test substances, the authors claim that the method has fair specificity and sensitivity compared to LLNA data and that the method is cheaper and quicker (4 versus 24 h incubation) than the DPRA (Auriault et al. 2011).

In silico methods

In silico models have been applied to predict the skin penetration potential and/or skin sensitizing potential of substances.

Quantitative structure–activity relationship (QSAR) models

Several QSAR tools contain modules for the prediction of skin sensitizers. None of them have achieved regulatory acceptance as stand-alone replacements to animal testing, especially in regard to the classification of non-sensitizers. So far, QSAR tools are being used for screening purposes and as one building block in a weight-of-evidence assessment. In case of a positive prediction and classification with GHS skin sensitization category 1A, the weight-of-evidence approach is unlikely to be challenged by regulatory authorities. However, so far, practical experience is scarce. Well-known tools containing a module for skin sensitization are DEREK NEXUS (Lhasa Inc), TOPKAT (Accelrys, Inc.) and MCASE (MultiCASE Inc). DEREK is a knowledge-based system using structural alerts and exclusion rules that can be combined with a module for general metabolism (METEOR). Upon positive prediction, detailed background information on reasoning is provided. TOPKAT is not rule based, but contains an algorithm for reactivity and information on whether a substance falls under the prediction domain of the model. It includes tools for validating the assessment by using univariate analysis and a patented so-called optimum prediction space method and specifically predicts sensitization as tested in the GPMT. In case of a positive prediction, it is possible to extract substances that are considered similar by the algorithm. MCASE is rule based with a hierarchy element for so-called biophores and modulators. MCASE can be coupled with a general metabolism tool (META). It should be emphasized

that, initially, all of these QSAR tools were established to serve the needs of the pharmaceutical industry, at a time when reliable data on skin sensitization for industrial chemicals were hardly available in the public domain. With increasing numbers of reliable experimental data on industrial chemicals and increasing interest of the chemical and cosmetic industry, the sensitization modules of these tools might be further developed. A more recent commercial tool called TIMES-SS was developed by OASIS Inc. TIMES-SS (TIssue MEtabolism Simulator for Skin Sensitization) is an expert system describing structure–toxicity and structure–metabolism relationships through a number of transformations simulating skin metabolism and interaction of generated reactive metabolites with skin proteins (Dimitrov et al. 2005). The skin metabolism simulator mimics the molecular transformation to be observed in the skin. The European Chemical Agency has put forward detailed descriptions of its requirements for reporting standards for QSAR predictions (Chapter 6 of the guidance on information requirements and chemical safety assessment; May 2008) based on the OECD Guidance document on the validation of (quantitative) structure–activity relationship models ([http://apli1.oecd.org/olis/2007doc.nsf/linkto/env-jm-mono\(2007\)2](http://apli1.oecd.org/olis/2007doc.nsf/linkto/env-jm-mono(2007)2)).

Within the EU project CAESAR, two QSAR models have been developed for assessing skin sensitizers from a data set of 209 known sensitizers and non-sensitizers. Validation, applicability domain and documentation standards were developed taking into account the ECHA reporting standards (Chaudhry et al. 2010). These rule-based models can be used free of charge at www.caesar-project.eu.

The developers of TIMES-SS were also involved in developing the protein-binding module of the OECD QSAR Toolbox, which is also available free of charge and was developed in recognition of the need for alternative methods under the REACH system. Version 2.3 of the OECD QSAR Toolbox was released in March 2012, and version 3.0 has been announced for October 2012. The OECD QSAR Toolbox contains a database of structural alerts for protein reactivity and a module for predicting metabolism in the skin. It identifies structural alerts and gives background information on the mode of action. In addition, it contains a continuously growing database on experimental data for substances. Therefore, skin sensitization can also be assessed by searching for substances of similar structure and/or by performing a trend analysis. The last update offers a module for the prediction of peptide binding potency. However, since only few of the known mechanisms for peptide binding have been investigated in regard to sensitization potency, this module frequently indicates that the target substance does not fall within the applicability domain.

Combinations of current methods

Without doubt, combinations of different *in vitro* methods will be required to properly predict the sensitizing properties of substances. In this context, it is worth noting that the OECD has drafted a manuscript in which the use of “adverse outcome pathways” (AOPs) is described (OECD: ENV/JM/MONO(2012)10). This concept foresees using mechanistic modes of toxicity to develop new methods, which reflect key steps that lead to adverse effects. In the case of sensitization, key steps of the AOP include skin penetration, peptide reactivity, DC activation, DC migration and T-cell proliferation. This entails that a combination of methods analyzing different endpoints will most probably be the most attractive approach. For example, in a method developed by CeeTox, peptide binding using glutathione depletion is combined with data from gene expression analysis of 11 genes, which are under control of the Keap1/Nrf2/ARE/EpRE, ARNT/AhR/XRE or Nrf1/MTF/MRE pathways. The system was challenged with 58 blinded substances, and the accuracy was found to be 84 %. An algorithm was used based on cell viability, chemical reactivity, the number of Nrf2 genes with mRNA levels over a certain threshold, the magnitude of gene expression and the exposure concentration that gave a response to enable potency categorization (McKim et al. 2010). Other combinations of methods were evaluated in an intralaboratory validation of the DRPA, h-CLAT, mMUSST, LuSens (an assay similar to the KeratinoSensTM assay) and the KeratinoSensTM assays (Bauch et al. 2012). Promising results were achieved when combining, for example, mMUSST, an ARE-based assay and the DPRA: With an overall accuracy of 94 % for the 54 test substances included in the study, there were no false-positive or negative predictions depending on the combinations of assays applied (Bauch et al. 2012). It remains to be seen whether the promising outcome of this testing strategy can be confirmed when extended panels of test substances from various industrial sectors are investigated. Jowsey et al. (2006) put forward an example for an integrated strategy for testing skin sensitizers and possibilities to determine relative potency of sensitizers. The strategy consists of 5 steps: (1) bioavailability, (2) structural alerts, (3) protein reactivity, (4) DC maturation and (5) T-cell proliferation. The scores from each individual test should be rated between 0 and 4 with a further score of 1 or 2 to be added (1) for substances having a structural alert from the *in silico* method and (2) for substances with low or high bioavailability. The scores from the five evaluations are then multiplied, resulting in an index of sensitizing potency. This ISP value forms the basis for categorizing the substances into non-sensitizers, weak, moderate, strong or extreme sensitizers. Although the concept of integrating

data from different *in vitro*, *in chemico* and *in silico* methods has been widely cited and suggested as the way ahead, the number of studies filling such a concept with data is still limited. In order to fill this gap, a combination of peptide reactivity, induction of ARE-dependent luciferase activity in a cell-based assay, TIMES-SS *in silico* prediction and calculated octanol–water partition coefficient was investigated by using novel as well as literature data on 116 substances. The results from the *in vitro* assays were scored 0–4 (non-sensitizer, weak, moderate, strong, extreme), and different calculations were performed. Using an optimized model, an overall accuracy of 88 % was obtained (Natsch et al. 2009). A more recent publication described the development of a so-called Bayesian network integrated testing strategy (BN ITS) to estimate skin sensitization hazard, which combines *in silico* (several parameters), *in chemico* (DPRA) and *in vitro* data (ARE luciferase activity, MUSST and IL-8 secretion) related to skin penetration, peptide reactivity and dendritic cell activation. It was concluded that a single generic set of test methods is unlikely to be the most effective and that the choice and order of tests depends on the information at hand and last but not least is chemical specific (Jaworska et al. 2011). Two similar approaches based on the concept of thresholds of toxicological concern (TTC) have been proposed, the dermal sensitization threshold (DST) based on LLNA data (Safford et al. 2008) and the threshold of sensitization concern (TSC; Keller et al. 2009). These tiered approaches for skin sensitization have been suggested where step 1 relates the identification of low-exposure substances, the threshold of sensitization concern (TSC). If human exposure to a given substance is expected to exceed TSC levels, it is suggested to perform a QSAR. If the result from the QSAR is negative, follow-up experiments using the DRPA are suggested. In case of doubt or uncertainties, a MUSST or h-CLAT assay should then be performed. The application of integrated testing strategies is also encouraged by the IVTIP association (De Wever et al. 2012).

Concluding remarks

Despite the fact that “Reduction” and “Refinement” has obviously been achieved for animal testing, the biggest challenge, “Replacement”, still remains to be achieved, even though considerable progress has been made. The LLNA method stands in line with two of the 3Rs, since it represents refinement in that the suffering of the animals is reduced in comparison with other the animal methods, and it often represents a reduction in animal use when compared to the GPTs. Despite the fact that the LLNA has been formally validated and is currently the method prescribed

for regulatory purposes, the Buehler test and the GPMT are still more frequently used for regulatory purposes worldwide than the LLNA (Rovida 2011). The major disadvantage of the conventional LLNA method is the use of radioactive labels although new non-radioactive methods are now available. Another study (Angers-Loustau et al. 2011) revealed that between the years 2001 and 2008, only 680 out of 3792 new chemicals registered with skin sensitization data had been tested in the LLNA although the percentage has risen in the last few years probably due to the adoption of the LLNA as OECD guideline 429 in 2002. These observations clearly demonstrate that the acceptance of new test methods takes time (Hartung 2009). Partially, this might be due to the years of experience and confidence assessors have in methods they have used for years. However, due to the increased demands from legislation as well as the increased interest in animal welfare from the public, promising in vitro methods are now being developed, which are based on key events in the sensitization process (i.e., skin penetration, protein or peptide binding, activation of LC/DC, T-cell proliferation, etc.). It is encouraging that three cell-based methods (MUSST, h-CLAT and the KeratinoSensTM assay) and one in chemico (DPRA) method have almost completed (pre-)validation at ECVAM.

An integrated approach, using a battery of methods covering the events in the sensitization process, will most likely be required to replace current animal models (Corcini et al. 2009; Jowsey et al. 2006; Dos Santos 2009). When assessing the sensitization potential of a substance for regulatory purposes, in particular with a focus on the 3Rs, a logical first step is to analyze the physico-chemical properties of the substance and, if available, also analyze human data. If the information is incomplete, in silico, in chemico and in vitro assays should be considered. In this context, a single method is not likely to be able to cover all steps of skin sensitization due to the complexity of the underlying mechanisms and the interactions between various cell types, mediators, chemokines, etc. The strengths and weaknesses of each assay will need to be identified in order to identify an optimal combination for predicting potential human allergens. Another important aspect to be kept in mind is that the LLNA data—the current “gold standard”—does not correlate 100 % with human data, so that comparison of in vitro methods to the LLNA is likely to multiply these errors if this not taken into account.

A European Commission expert group has estimated that it will take another 7–9 years before the current in vivo animal tests for skin sensitization can be fully replaced (Adler et al. 2011). Criticism to that timescale has been raised by the authors as it does not include the time it takes for regulatory acceptance and will not be achievable for all

chemicals. Considering the existence of QSAR models accepted for regulatory purposes for REACH in combination with the fact that three alternative methods (DPRA, MUSST and h-CLAT) have almost completed (pre-)validation, Taylor et al. (2011) claimed that current animal models for testing skin sensitization can be replaced by earlier time points. An expert consortium review also suggested that the methods for predicting sensitization potential might become available within considerable shorter time frames than 7–9 years (Hartung et al. 2011). These estimations might not have taken into account a number of unresolved scientific problems related to the application in vitro methods, for example, for the testing of solid substances, substances with low water solubility or those that are very cytotoxic and the time required for regulatory acceptance.

No toxicity test is completely perfect. Each method requires a balance between sensitivity and specificity. These are two very different aspects, and tests can be developed to cater to either the one or the other—or to overall accuracy, depending on the context in which the test is used (e.g., regulatory setting, screening and/or different stages in product development). One single assay may be optimal for a certain class of substances but can at the same time be suboptimal for chemicals of other classes.

Another aspect is that the topically applied dose of a substance may not reflect the actual dose in the dermis. The stratum corneum of the skin is a natural barrier for the entry of substances. Current in vitro cell culture models do not consider this barrier, which contributes to the reduced bioavailability of substances in vivo. To overcome this deficiency, testing strategies may need to be developed that allow evaluating dermal penetration on in vitro skin models and/or that include in silico evaluations such as QSARs, to enable quantitative correlation of the chemical structure of a substance to its dermal penetration (Basketter et al. 1999; Pendlington et al. 2008).

Keeping in mind that an integrated test strategy will only be meaningful if the methods included cover a broad variety of different endpoints, it is very likely that by using a combination of methods, the majority of the limitations addressed above will be overcome. Although reliable non-animal methods for the prediction of sensitization hazard are within reach, at least for a large number of chemicals, potency estimation is still an unresolved issue for risk assessment when using the currently available in vitro methods (Basketter and Kimber 2010). Finally, most of the data generated from in vitro alternative methods so far derive from experiments with single substances. To properly predict the skin sensitization hazard of mixtures and, more importantly, their potency will be an additional challenge.

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