



Review

Identifying and characterizing chemical skin sensitizers without animal testing: Colipa's research and method development program

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ABSTRACT

The sensitizing potential of chemicals is usually identified and characterized using one of the available animal test methods, such as the mouse local lymph node assay. Due to the increasing public and political concerns regarding the use of animals for the screening of new chemicals, the Colipa Skin Tolerance Task Force collaborates with and/or funds research groups to increase and apply our understanding of the events occurring during the acquisition of skin sensitization. Knowledge gained from this research is used to support the development and evaluation of novel alternative approaches for the identification and characterization of skin sensitizing chemicals. At present one *in chemico* (direct peptide reactivity assay (DPRA)) and two *in vitro* test methods (cell based assays (MUSST and h-CLAT)) have been evaluated within Colipa inter-laboratory ring trials and accepted by the European Centre for the Validation of Alternative Methods (ECVAM) for pre-validation. Data from all three test methods will be used to support the development of testing strategy approaches for skin sensitizer potency prediction. The replacement of the need for animal testing for skin sensitization risk assessment is viewed as ultimately achievable and the next couple of years should set the timeline for this milestone.

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Abbreviations: ACD, allergic contact dermatitis; DC, dendritic cells; DNCB, 2,4-dinitro chlorobenzene; ECVAM, European Centre for the Validation of Alternative Methods; KC, keratinocyte; LC, langerhans cells; LLNA, local lymph node assay; PBL, human peripheral blood leucocytes; PBMDs, human peripheral blood monocytes derived DCs; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TCPA, T cell priming assay; TCR, T cell receptor.

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1. Introduction

Skin sensitization is the induction of an allergic immune response following skin exposure that can be induced by a subset of chemicals. Allergic contact dermatitis (ACD) is the clinical condition resulting from skin sensitization which is a delayed-type hypersensitivity reaction induced by small reactive chemicals (haptens) (Smith and Hotchkiss, 2001). Guinea pig models were historically used to identify whether a chemical has the potential to induce skin sensitization in humans. More recently, a refined and reduced method, the murine local lymph node assay (LLNA) has been employed (Basketter et al., 2002) and the sensitizer potency information generated can be used to predict a safe level of human exposure (using a Quantitative Risk Assessment approach (Felter et al., 2002, 2003)). There are, however, increasing public and political concerns regarding the use of animal testing for the screening of new chemicals. Consequently, the development of *in vitro*, *in chemico* or *in silico* models for predicting the sensitizing potential of new chemicals is receiving widespread interest. These tests will need to resume the complex interactions of a chemical with the different compartments of the immune system: The chemical must penetrate the skin and react with endogenous proteins. Some chemicals, termed pro-haptens, require activation through skin metabolism in order to become haptens capable of binding to skin proteins. Haptenated carrier-proteins are internal-

ized and processed by immature dendritic cells (DCs) that become activated. The activated DCs start to migrate from the epidermis into the draining lymph node, complete maturation and present fragments of the haptenated carrier-proteins to T-helper cells, resulting in an antigen-specific immune response.

Colipa, the European trade association representing the interests of the cosmetic, toiletry and perfumery industry consists of 25 national associations (representing over 2000 small and medium enterprises), 18 major international companies, four supporting associations and two correspondent members currently collaborates with several academic and industrial research groups to expand our understanding of the molecular and cellular events occurring during the acquisition of skin sensitization (Basketter et al., 2005; Colipa, 2007; Aebly et al., 2008; Maxwell et al., 2010). At present fundamental and applied research is being funded in multiple key areas, such as modeling of skin bioavailability, hapten chemistry, peptide binding, skin metabolism, dendritic cell activation and T cell proliferation (see Fig. 1). Knowledge gained from this research is being used to support the development and evaluation of novel alternative approaches for the identification and characterization of skin sensitizing chemicals. The current replacement rationale for skin sensitization is that data from several non-animal test methods will need to be combined as part of a testing strategy to predict skin sensitizer potency information (Jowsey et al., 2006; Maxwell et al., 2008; Natsch et al.,

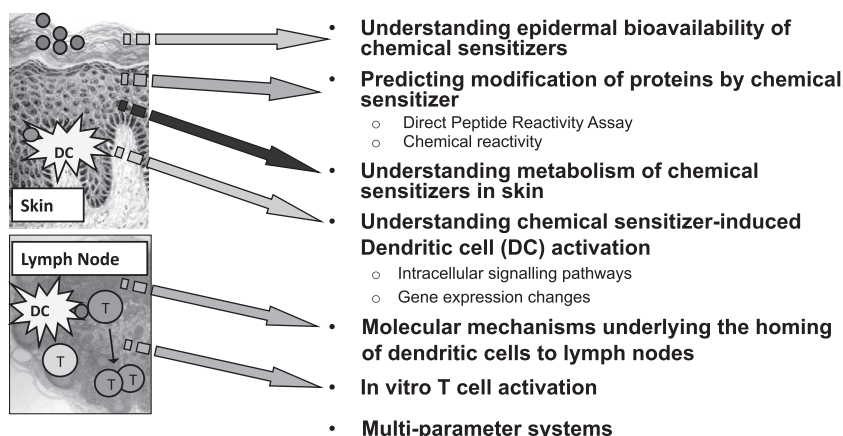


Fig. 1. Overview of the Colipa research activities.

2009). At present one *in chemico* (direct peptide reactivity assay (DPRA)) and two *in vitro* test methods (cell based assays (MUSST and h-CLAT)) have been evaluated for their potential to predict skin sensitization potential within Colipa inter-laboratory ring trials and accepted by the European Centre for the Validation of Alternative Methods (ECVAM) for pre-validation.

This publication aims to provide an overview and a progress report on all Colipa-funded research, method development and method evaluation activities, as well as presenting a cosmetic industry viewpoint on the feasibility of replacement of animal testing for skin sensitization.

2. Skin exposure and bioavailability

The extent and frequency of skin exposure to a sensitizing chemical has long been known to play a key role in determining the potential for and severity of ACD (Upadhye and Maibach, 1992; Basketter et al., 2006). Exposure information for various product types has been generated and is routinely used to inform consumer safety risk assessments (e.g. Loretz et al., 2005, 2006, 2008). However as non-animal test methods are adopted there is an increasing need to understand skin exposure at the cellular and molecular level to enable appropriate interpretation and extrapolation of *in vitro* or *in chemico* data. Consequently, Colipa has invested in exploring the application of new technologies for the prediction of skin bioavailability and for evaluating the role of metabolism in skin sensitization.

2.1. Toxicokinetic model for prediction of epidermal bioavailability

In vitro sensitization tests need to resolve the complex interactions of a chemical with the different compartments of the immune system. As a first step, in order to have sensitizing potential a chemical must penetrate the skin barrier and reach the viable part of the epidermis. In order to better understand this process and to be able to make assumptions on the epidermal bioavailability of new test chemicals, the group of J. Kasting at the University of Cincinnati, USA has developed, with the support from Colipa, a toxicokinetic model to allow a more accurate prediction of the epidermal bioavailability of potential skin sensitizers. This computer model of skin absorption is based on physico/chemical data of the skin: The stratum corneum is modeled as a two-phase membrane with lipids and corneocytes. The dermis is a fiber matrix with distributed capillary clearance and the viable epidermis is treated as unperfused dermis. The model takes into account finite dose application where the dosing regime can be adapted (single or multiple dosings) and will initially simulate the LLNA dosing regime. The model predicts key skin parameters such as; C_{max} (the peak mid epidermal concentration of freely diffusing permeant); AUC_{120} (the area under the concentration-time profile of freely diffusing permeant 120 h after dosing) and percentage of dose absorbed. A preliminary model (based around an Excel spreadsheet interface) has been developed and is now available for use. As inputs, the physico/chemical, structural and skin properties and dosing scheme are required. The system predicts the concentration of chemical in the viable epidermis over time (C_{max} , AUC_{120} , T_{max} , C_{free} , % absorbed, etc.) and data has already been generated for >200 chemicals.

2.2. Modification of skin proteins by chemical sensitizers

A prerequisite for the occurrence of both antibody- and cell-mediated allergic reactions against chemicals is the activation of chemical-specific T cells. Chemical-specific T cells do not recognize the chemical itself but instead recognize a conjugate of the chem-

ical (hapten) in the context of a protein fragment (a peptide) that can be presented to T cells by class I or class II MHC molecules on the surface of antigen-presenting cells, such as Langerhans' cells (LCs). Chemical sensitizers can either act as a hapten (i.e. chemical is inherently reactive and will covalently bind to amino acid side chains) or as a pro-hapten (chemical requires metabolic or chemical conversion to a protein-reactive species). Since a correlation between protein reactivity and skin sensitization has been established, the measurement of the protein binding properties of a chemical may represent a promising endpoint for the development of an alternative approach to animal testing (Gerberick et al., 2008).

2.3. Direct peptide reactivity assay (DPRA)

The DPRA protocol was developed with the support of Colipa by Procter & Gamble, Cincinnati, USA in collaboration with the group of J.P. Lepoittevin at the University of Strasbourg, France (Gerberick et al., 2004, 2007). The test method aims to model protein haptenation *in chemico*, by measuring the depletion of two synthetic peptides (containing either a single cysteine or lysine as a reaction target) using High Performance Liquid Chromatography. The prediction model for the DPRA uses a classification tree approach to assign different reactivity categories to the depletion data from the cysteine and lysine synthetic peptides (Gerberick et al., 2007). Using this approach the DPRA had a 89% concordance with LLNA potency categories (i.e. weak, moderate etc.) and following two rounds of ring trials inter-laboratory reproducibility was also found to be acceptable. Consequently, the current protocol has been transferred to ECVAM for phase 3 pre-validation (Hartung et al., 2004).

In addition, Colipa continues to support the ongoing collaboration between Procter & Gamble and the University of Strasbourg that is currently focused on increasing the applicability domain of the current test method to include poorly soluble chemicals and pro-haptens that require oxidation to become active (Gerberick et al., 2009).

2.4. Skin innate immune response: construction of a multi-cell human skin model

Keratinocytes are the predominant epidermal cell type and are known to play a key role in the activation of LCs in response to a pathogenic stimulus or physical/chemical stress (Cumberbatch et al., 1992; Griffiths et al., 2005). Although keratinocytes in isolation are unlikely to be directly predictive of skin sensitization hazard, Colipa remains interested in the role that epidermal keratinocytes and/or dermal fibroblasts cells could play in the activation of LCs and dermal DCs. Consequently a project was recently identified for funding that aims to integrate these sensitizer-induced innate immune signals using novel technologies. This project is in collaboration with the groups of A. Ghaem Maghami at University of Nottingham, UK and J. Haycock at University of Sheffield, UK. It aims to engineer a multi-cell skin model comprising human dendritic cell-like cells, co-cultured with primary human keratinocytes in the presence of human dermal fibroblasts, within a perfusable multi-cell environment, that is amenable to interactive control and monitoring of cell emergence, maturation and interaction. This system will also allow for the co-culture of conditioned DC-like cells with T cells and evaluation of DC-induced T cell differentiation. It is anticipated that such a model will efficiently simulate the natural microenvironment in which these cells co-exist, and therefore once established it may be an effective *in vitro* tool for the prediction of allergenic potential of novel chemicals, studying the mechanisms of chemical induced skin sensitization

and the definition of the role of other cutaneous cell types in this process.

2.5. Characterization of the metabolic capacity of human skin and of relevant *in vitro* test systems

Reconstructed skin models have become a standard in testing toxic effects. Because of their physiological resemblance to native skin they have been included into test systems for the identification of sensitizing compounds. To ensure the data generated by *in vitro* test methods can be applied appropriately for risk assessment decision-making the metabolic capability of these test systems has to be characterized thoroughly.

Consequently Colipa is currently working with the groups of E. Fritsch at the University of Duesseldorf, Germany and R. Edwards at Imperial College London, UK to evaluate the metabolic competency of human skin relative to *in vitro* human epidermal equivalent and immortalized keratinocyte models. The Imperial College project aims to profile the enzymes of the xenobiotic metabolism by means of mass spectroscopy followed by immunochemistry and focuses on the protein expression profile of native skin and an *in vitro* epidermal equivalent. Antibodies have been established for human cytochrome P450s (CYPs) 1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, 4A11 and GSTpi isoforms. Feasibility and subsequent raising of antisera to other CYPs, prostaglandin H-synthase (PGHS), glutathione-S-transferase (GST), UDP-glucuronosyltransferase-activity (UGT), and N-acetyl-transferase (NAT) isoforms will be performed as part of the project.

The University of Duesseldorf project focuses on the characterization of basal and inducible enzyme activity of phase I enzymes like CYP1A and CYP1B. CYP2E1, CYP2B and CYP3A will be analyzed as well. Moreover, important phase II enzyme will be characterized like: GST- and PGHS-activities, UGT-activity and NAD(P)H-Quinone reductase-activity.

Differences with respect to the expression of CYPs can be observed between excised human skin, HaCaT and the epidermal equivalent and further comparative studies are on the way.

2.6. Sensitizer-induced DC activation

The next biological step in the sensitization process is the internalization and processing of the haptenated self-proteins by immature DCs. During this process DCs mature to an activated state i.e. up-regulate the expression of a set of cell surface markers (e.g. co stimulatory molecules such as CD83, CD86 or adhesion molecules such as CD54), secrete various cytokines such as IL-1 β , IL-8 and down-regulate proteins involved in antigen uptake such as aquaporins. DCs, whose central role during the induction process of skin sensitization is well documented, were perceived as a favorable opportunity for developing *in vitro* approaches for detecting potential sensitizers. Recent advances in the *in vitro* generation of immature DCs and the availability of various cell lines with DC-like phenotypes have led to the development of many *in vitro* protocols for measuring the activation of DC-like cells upon exposure to chemicals.

2.7. DC activation tests using human peripheral blood monocytes derived DCs

Different research groups have published *in vitro* test protocols based on PBMDs (see Dos Santos et al. (2009) for review). In the protocol proposed by one member of the Colipa Skin Tolerance Task Force (see Aebly et al., (2004), PBMDs were exposed for 3–30 h to the test chemicals. DC maturation was evaluated by flow cytometric measurement of the percentage of CD86 positive cells and quantitative measurement of the mRNA expression of inter-

leukin-1 β , interleukin-8 and aquaporin 3 using real time PCR. This approach has been used successfully to analyze the sensitizing properties of many reactive chemicals and has been used for a detailed analysis of the sensitizing properties of p-phenylenediamine (PPD) through its oxidation products and possible inhibition by acetylation in the skin (Aebly et al., 2009). The authors concluded that the described *in vitro* test system allowed a refined analysis of the sensitizing properties of chemicals and could further improve product safety.

3. DC activation tests using cell lines

3.1. U937

Major drawbacks of peripheral blood derived DCs are their complex and expensive preparation procedures, sourcing issues and their donor-to-donor variability. As a possible alternative, human myeloid leukemia cell lines were perceived as possible DC surrogates. Therefore, Colipa has supported a project for the development and evaluation of an *in vitro* test system using the human myeloid cell line U937 for the detection of contact allergens. This effort resulted in the preparation and publication of a test protocol (Python et al., 2007). Briefly, cells are seeded in 12-well plates and treated with the test chemicals for 24 h, 48 h and 72 h. The cells are analyzed by flow cytometry for CD86 surface expression and cell viability. In parallel, IL-1 β and IL-8 gene expressions are measured by quantitative real-time RT-PCR. The biological response for each tested chemical is evaluated by considering modulation of the three selected activation markers (CD86, IL-1 β and IL-8) at each time point. The results suggested that a chemical inducing a significant up-regulation of the expression of at least two markers might be considered as a sensitizer. In that first phase, the described test system (U937 activation test) was able to correctly classify 15 out of 16 tested chemicals.

3.2. Myeloid U937 skin sensitization test (MUSST)

Protocols based on U937 cell line were further developed by L'Oréal (Ade et al., 2006) and Cosmital SA, Procter & Gamble (Python and Aebly, see above) within the Colipa framework. Historically, L'Oréal has determined that the assay's concordance with human clinical data was ~85% (based on 99 reference chemicals).

In a first phase, the MUSST protocol based on L'Oréal's original procedure was distributed within Colipa members and evaluated in the L'Oréal, Cosmital and LVMH laboratories (for a schematic outline of the MUSST protocol, see Fig. 2). Briefly, U937 cells are exposed for 48 h in 96 well plates to a concentration range of chemical. CD86 expression and cell viability (propidium iodide

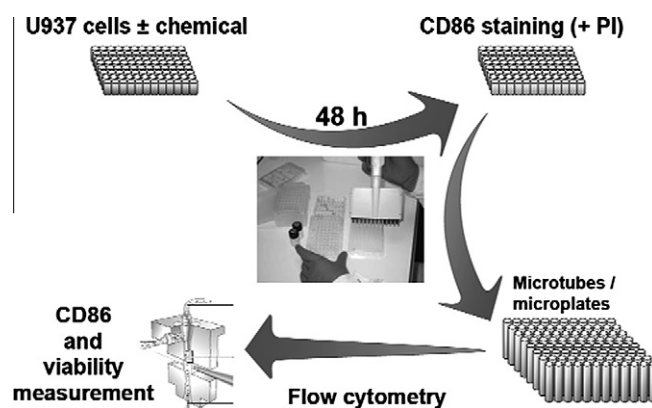


Fig. 2. Outline of the MUSST protocol.

exclusion) are then measured by flow cytometry. A chemical is predicted sensitizer if it induces a dose-dependent increase of CD86 expression at non toxic doses (viability > 70%) within two concordant experiments.

After some further optimization (cell density, markers measurement, etc.), the protocol was transferred into two further laboratories (Shiseido and Beiersdorf) for the evaluation of test transferability and robustness. L'Oréal also assessed the predictive capacity of the MUSST using the finalized protocol with 40 chemicals (21 sensitizers and 19 non-sensitizers). The overall performance of the MUSST was very encouraging and the results supported the successful submission of the test to ECVAM to enter a phase III pre-validation.

3.3. THP-1

The human monocytic leukemia cell line THP-1 was first derived from the peripheral blood of a 1-year-old male with acute monocytic leukemia (Tsuchiya et al., 1980, 1982). These cells can be obtained through major cell depositories including the American Type Culture Collection (ATCC). They are easy to culture and have been widely used in various studies. Following PMA treatment, the THP-1 cells differentiate to macrophages.

During LC/DC activation, modulation of cell surface molecules (e.g. CD86, CD54, CD40, and CD83) and secretion of pro-inflammatory cytokines such as TNF- α , IL-8, IL-1 α and IL-6 are observed (Cumberbatch and Kimber, 1992; Enk and Katz, 1992; Cumberbatch et al., 1996; Ozawa et al., 1996; Verrier et al., 1999). In THP-1 cells, CD40, CD54, CD80, CD83, CD86, and HLA-DR were reported to be inducible cell surface markers. Among these markers, many researchers reported that CD86 (Ashikaga et al., 2002; Yoshida et al., 2003; Tietze and Blomeke, 2008; Ade et al., 2009) and CD54 (Yoshida et al., 2003; Ashikaga et al., 2006; Sakaguchi et al., 2006; Mitjans et al., 2008) were up regulated after treatment with many allergens. MHC class II molecule internalization (Ashikaga et al., 2002), CD1a, CD40 and CD80 induction (Miyazawa et al., 2007) were also observed with some allergens. The secretion of the chemokine MIP-1 β (Hirota and Moro, 2006; Lim et al., 2008) or of the cytokines IL-1 β (Yoshida et al., 2003), TNF- α and IL-6 were also proposed as activation markers in THP-1 cells. Recently, increases in the production of IL-8 (Mitjans et al., 2008; Nukada et al., 2008) or phosphorylation of the p38 MAPK in allergen stimulated CD34-DCs (Boislevé et al., 2004) and THP-1 cells (Miyazawa et al., 2008; Mitjans et al., 2008) were also reported.

These data suggest that the response of THP-1 cells is similar to that of LC/DC after exposure to allergens. THP-1 cells therefore represent promising substitute for the development of an *in vitro* skin sensitization test.

3.4. The human cell line activation test (h-CLAT)

The h-CLAT protocol, using THP-1 cells, was developed through a cooperation of two Japanese companies: Kao and Shiseido (Sakaguchi et al., 2006; Ashikaga et al., 2006). The outline of the h-CLAT protocol (see Fig. 3) is the following: THP-1 cells are pre-cultured for 48 h or 72 h and exposed to the test chemical for 24 h. The expression of two cell surface antigens, CD86 and CD54, is then measured by flow cytometry. Two of three independent measurements at any dose should exceed the positive criteria (CD86 $\geq$ 150% or CD54 $\geq$ 200% of their respective vehicle control) in order to be judged as positive. The concordance with LLNA results was about 84% (Ashikaga et al., 2010). There are several reports describing the activation mechanism of THP-1 cells (e.g., Y. Miyazawa et al., 2008), and publications that support the protocol of the assay (e.g., M. Mizuno et al., 2008). Ring trials between Colipa members (P&G, Henkel, L'Oréal, Kao and Shiseido) and Japanese

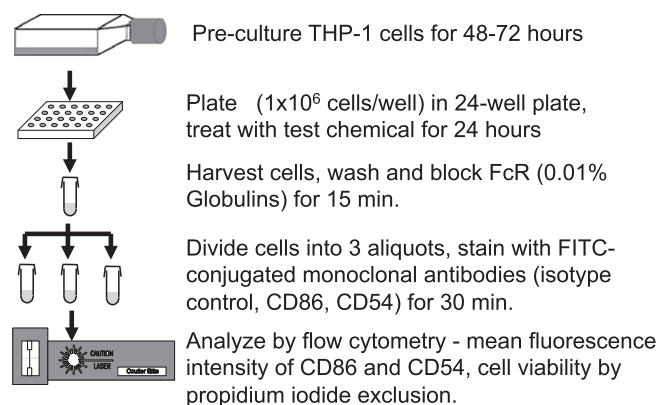


Fig. 3. Outline of the h-CLAT protocol.

cosmetic companies (Kanebo COSMETICS, Nippon Menard Cosmetics, Shiseido, Kao, KOSE and LION) respectively have been conducted for several years. Issues could be identified and the protocol and its prediction model further optimized. The latest results showed that the h-CLAT protocol is easy to transfer and that inter-laboratory reproducibility is acceptable (Ashikaga et al., 2008; Sakaguchi et al., 2010). These data also supported the submission of the h-CLAT into a formal pre-validation process and the current protocol has been accepted by ECVAM for phase III pre-validation.

3.5. Intracellular signaling pathways

The h-CLAT or MUSST protocols measure the activation of DC-like cells after exposure to sensitizers. However, the early intracellular mechanisms involved in DC maturation are still not well understood. To address this knowledge gap, Colipa has initiated a research project with the group of M. Serres, Hôpital E. Herriot, Lyon, France, where DCs derived from human monocytes are treated with sensitizers (nickel, DNCB or thimerosal) in comparison with an irritant (SDS). Sensitizers induced the up-regulation of DC activation markers (CD86, CD54) and cytokine secretion (IL-8 and TNF α) whereas SDS had no effect. Kinase activity measurements (Face™ method and CBA) showed no effect of SDS. On the other hand, NiSO₄ activated JNK, Erk1/2 and p38 MAP kinase (MAPK), whereas DNCB and thimerosal only activated p38 MAPK and JNK, but markedly inhibited Erk1/2. A pre-treatment with p38 MAPK inhibitor (SB 203580) suppressed Erk1/2 inhibition induced by DNCB or thimerosal demonstrating a direct interaction between p38 MAPK and Erk1/2. Moreover, the role of reactive oxygen species (ROS) on MAPK activation was demonstrated using a pre-treatment with an antioxidant, N-acetyl-L-cysteine (NAC), which reduced Erk1/2 inhibition, induced by DNCB and thimerosal, and markedly reduced p38 MAPK phosphorylation suggesting a direct activation of p38 MAPK via an oxidative stress. Thus, the regulation of MAPK signaling pathways appears to vary between chemical sensitizers (Trompezinski et al., 2008).

To better understand the role of p38 MAPK and Erk1/2 in DC activation, these studies were performed with an extended list of chemicals including (according to the LLNA classification) extreme sensitizers such as 4-nitrobenzyl bromide (NBBBr) and MCI/MI with DNCB as positive control, moderate sensitizers such as cinnamic aldehyde and tetramethyl thiuram disulfide (TMTS), and cinnamic alcohol as weak sensitizer. Non-sensitizers such as lactic acid, glycerol and salicylic acid or SDS as an irritant were also evaluated. All of these chemicals were investigated on mono-DCs and with the U937 and THP-1 (h-CLAT protocol) cells. These cell lines being non-adherent, the Face™ method had to be adapted and opti-

mized. Data on kinase show that in mono-DCs all sensitizers markedly activated p38 MAPK and inhibited Erk1/2, many of them via a predominant role of oxidative stress (Trompezinski et al., 2008). Similar data were obtained with the U937 cell line whereas the THP-1 line responded differently in particular on Erk1/2 pathway which is always increased but neither inhibited as also reported by Miyazawa et al. (2008). During all these experiments, non-sensitizers and irritants did not influence kinase activities. On the other hand, an up-regulation of CD86 expression and an increase of IL-8 secretion were detected with all sensitizers and all types of cells used. It is important to note that weak and moderate sensitizers needed 10× and 100× higher exposure concentrations respectively than strong sensitizers to activate kinase pathways.

Since oxidative stress plays a major role in kinase regulation, the possible role of calcium influx as an early event in DC activation was also investigated. Preliminary data demonstrated that an ionophore such as A23187 or ionomycin markedly upregulated CD86 and IL-8 secretion both in mono-DCs and U937 and THP-1 cell lines. A calcium influx is detected (fluorescent probe Fluo8™ AM from Molecular Probes) in mono-DCs and both cell lines after exposure to all tested chemicals previously mentioned. As reported for kinase pathways, a pre-treatment of cells with an antioxidant, NAC, markedly decrease this calcium influx confirming a major role of an oxidative stress in DC activation and particularly of SH groups as other antioxidants non thiol-dependent were without effect (vitamin C, vitamin E and aspirin). The calcium influx was detected around 3 h after cell exposure to chemicals, suggesting that calcium influx is a second event following ROS induction.

The key role of mitochondria, a “reservoir” of ROS in DC activation was demonstrated using mitochondrial specific fluorescent probes to measure ROS (MitoSox from Fluoroprobes) or membrane depolarization (JC-1 from Fluoroprobes). All these studies highlight the essential role of SH groups but not antioxidant non thiol-dependent in DC activation via ROS, kinase and calcium signaling.

In perspectives, a more complete investigation of the crosstalk between kinase activities, calcium influx and an oxidative stress as early events in DC activation induced by sensitizers is required using several sensitizers, non-sensitizers and more irritants. Some of these questions are in part addressed in a new research project that COLIPA has initiated recently: The groups of M. Pallardy (INSERM UMR-S 749, Université Paris-Sud, France) and J.-P. Lepoittevin (CNRS UMR 7177, Université de Strasbourg, France) will address jointly the question of the mechanistic link between chemical reactivity measured *in chemico* using model peptides and chemical stress induced in DC, by measuring Nrf-2 activity *in cellulo*. Moreover, these two parameters will be analyzed in relation to other known DC activation markers (danger signals, MAPK phosphorylation, CD86 expression, IL-8, ROS (MitoSox) calcium influx, etc.).

3.6. Gene expression changes predicting DC activation potential

In order to identify DC genes that are modulated by exposure to allergens Colipa has funded a research project aiming towards the development of a high throughput method to screen for gene expression changes for the prediction of the skin sensitization potential of chemicals. In a first step, the effect of exposure of human monocyte derived DC (MoDC) to a contact allergen was examined at the transcriptional level using Affymetrix GeneChip®. This analysis revealed 173 genes that are significantly modulated (Ryan et al., 2004). Using a test set of 15 chemicals, 29 of these genes were selected for further evaluation of predictive potential using RT PCR (Gildea et al., 2006). In order to develop a high throughput method to measure the expression of this set of genes the Luminex® xMAP® platform-based QuantiGene® Plex assay was employed as a suitable high throughput method for detecting changes in

gene expression. It permits the examination of many genes in single sample at the same time.

In work conducted with PBMDc, very little donor-to-donor variability was observed in the gene expression changes induced by treatment with 5 mM 2,4-dinitrobenzene sulphonic acid (DNBS), with 13 out of 29 genes consistently positive in a total of 27 donors. Excluding DNBS, no gene expression changes were consistently positive with all of the allergens tested. It was observed that some allergens induce changes in only a few genes.

Compared to PBMDc, the DC-surrogate cell lines evaluated showed fewer changes in the expression of the 29 genes following allergen treatment, especially the U937 cells. MUTZ-3 and THP-1 cells were found to be slightly more responsive than U937 cells. In addition, all three cell lines were similar to PBMDc in their response to non-allergens. The inter-experimental variability noted in the response of the cell lines would indicate the need for repeated independent experiments, as is the case for other cell line-based *in vitro* methods for skin sensitization testing (e.g. h-CLAT and MUSST).

Using changes in the expression of these 29 genes as an indicator of DC activation by allergens, none of the DC-surrogate cell lines (THP-1, U937 and MUTZ-3) tested appeared to be a suitable replacement for PBMDcs based on the results of the pilot set of chemicals. However, further testing of an expanded chemical test set using the most promising genes identified in this work may reveal the appropriateness of a DC-surrogate cell line for this purpose.

3.7. Identification and exploitation of molecular mechanisms underlying the homing of DCs to lymph nodes

Following DC maturation the subsequent migration of DCs to the lymph nodes is obligatory for the resulting sensitization to happen. A key process in this migratory step is the up-regulation of a chemokine receptor known as CCR7 which allows the DCs to leave the tissue and follow the trail of the CCR7 chemokine ligands, CCL19 and CCL21, produced by the lymphoid tissue. Consistent with the notion that CCR7 is a key molecule for the homing of mature DCs to the lymph node, mice deficient in CCL19, CCL21 or CCR7 have a greatly impaired capacity for the recruitment of DCs to draining lymph nodes. Different chemical sensitizers are likely to induce a variety of responses from DCs, which directly or indirectly promote skin sensitization. The process of CCR7 up-regulation is postulated to be a hub at which these different pathways are likely to converge. Consequently a research project proposed by J.E. Pease, Imperial College London, UK, was recently identified for funding. It aims to identify and characterize the portion of genomic DNA responsible for optimal CCR7 gene induction in leukocytes. This will be used to generate a reporter gene driving luciferase expression in response to CCR7 up-regulation and to use that recombinant cell line to assess the sensitizing potential of a panel of chemicals.

3.8. Multi-parameter DC biosensor system to identify contact allergens

Colipa is supporting the group of A. Takashima, University of Toledo, Ohio, USA in the further development and evaluation of a combined DC biosensor system. It is composed of a DC-based transcription factor profiling system and a keratinocyte (KC)-based ATP release assay. Briefly, the XS106 DC-line was engineered to express the yellow fluorescence protein (YFP) reporter gene under the control of IL-1β promoter. The resulting DC biosensor clone has a high sensitivity and broad reactivity to all tested stimuli known to induce LC maturation (Mizumoto et al., 2005). Secondly, to study molecular mechanisms controlling DC activation in a more systematic fashion, the XS106 DC were transduced to express the lucifer-

ase (Luc) reporter gene under the control of each of 15 different cis-enhancer elements. The resulting DC-based transcription factor profiling system is able to discriminate different stimuli by activating distinct sets of gene transactivation pathways (Mizumoto et al., 2005). Third, based on the finding in CD39-deficient mice that ATP released from chemically injured KCs serves as a causative mediator of both irritant and allergic contact dermatitis (Mizumoto et al., 2002), a KC-based ATP release assay was developed. A small-scale validation study with structurally diverse skin irritant compounds demonstrated relatively high sensitivity (false negative rate = 0/18) and specificity (false positive rate = 1/20) of the KC-based *in vitro* assay (Mizumoto et al., 2003). The aims of the collaboration with Colipa are to evaluate the practical utility of this multi-parameter *in vitro* cell-based biosensor system by testing a well-characterized panel of contact allergens and to further develop newer generations of DC-based *in vitro* assay platforms to detect a full spectrum of contact allergens.

4. T cell proliferation

4.1. T cell priming assay

Another way to detect the sensitizing properties of a chemical is to demonstrate that it can induce priming of specific T cell precursors. To develop *in vitro* models of sensitization, several laboratories have used different systems involving normal human peripheral blood leucocytes (PBL) and mature or immature DC to induce *in vitro* sensitization of T cells with different readouts, e.g. proliferation or cytokine production (Krasteva et al., 1996; Rustemeyer et al., 1999; Rougier et al., 2000). Although some strong experimental haptens were able to activate T cells in these primary sensitization assays, the magnitude of the T cell response was routinely low and the results were poorly reproducible. More importantly, attempts to develop such *in vitro* T cell sensitization assays for common weak haptens have failed so far. For the past 2 years Colipa has collaborated with the group of J.F. Nicolas at INSERM in Lyon, France to develop a human DC: T cell co-culture approach based on a protocol that was originally developed using murine tissues (Vocanson et al., 2006). The TCPA protocol is based around co-culturing sensitizer-haptenated, irradiated monocyte derived DC with T cells that have been depleted for the CD25 + regulatory T cell subset. The rationale, which proved successful in the mouse system, is that removal of the regulatory T cell fraction will lower the threshold at which a T cell proliferative response can occur. In so doing, the sensitivity of the test method for weak and moderate sensitizers increases relative to standard DC: T cell co-culture protocols. It could be demonstrated that depletion of PBLs in CD4 + CD25 + Treg cells (using CD25 + microbeads and cell magnetic separator) significantly increased hapten-specific T cell priming against numerous contact allergens and notably weak allergens (2,4,6-trinitrobenzenesulfonic acid (TNBS), fluorescein isothiocyanate (FITC), hexylcinnamaldehyde, dihydrocoumarin, paraphenylenediamine, methylidibromoglutaronitrile, isoeugenol, eugenol, etc.) indicating that the human TCPA (hTCPA) is able to detect the immunogenic properties of haptens. Nevertheless, the sensitivity and specificity of the current hTCPA is not optimal. Indeed, for weak allergens, positive responses were obtained in only 1 out of 2 experiments. Moreover, some sensitizers (cinnamaldehyde, glyoxal) failed to induce T cell priming and some false positive responses were also observed (lactic acid, salicylic acid). Therefore, improvement of the hTCPA sensitivity and specificity is being evaluated by using PBLs fully deficient in regulatory cells (Luci and Tomasello, 2008) and monocyte-derived dendritic cell subsets with potent priming activities (Tsushima et al., 2007).

A related approach is based on the hypothesis that the strength/potency of contact allergens is reflected by the effector and regula-

tory T cell frequency as well as by the diversity of the T cell receptor (TCR) repertoire (Kotturi et al., 2008; Moon et al., 2007; Dietz et al., in preparation). Colipa has initiated a collaboration with the group of S. Martin, Department of Dermatology, University Medical Center, Freiburg, Germany for the development and evaluation of an *in vitro* T cell priming assay. In this assay naive T cells will be primed with dendritic cells that are directly modified with contact allergens of different potencies according to LLNA EC3 values or will be loaded with hapten-modified human serum albumin. T cell IFN-gamma production as well as the upregulation of activation markers will be used to determine the frequency of the contact allergen-primed effector T cells. The frequencies will be correlated with the T cell receptor repertoire analysed by TCR spectratyping of the contact allergen specific T cells isolated based on IFN-gamma. This *in vitro* T cell priming assay should yield information on the frequency of effector cells generated during sensitization to contact allergens and the possible correlation of the potency with the TCR repertoire diversity.

5. Discussion

Colipa continues to fund a range of research and method development activities with the aim of delivering a 'tool box' of *in silico* and *in vitro* test methods that will be capable of identifying and characterizing the potency of skin sensitizers when used in concert. In this way, we aim to remove the need for new animal test data when making future skin sensitization consumer safety risk assessment decisions. This review demonstrates the progress made during the last few years whilst also highlighting the challenges ahead.

Most notably our ability to make risk assessment decisions using non-animal test method data has still to be evaluated and despite some intriguing theoretical (e.g. Jowsey et al., 2006) and applied case studies (e.g. Natsch et al., 2009) the challenge of how to apply a non-animal test battery remains open to debate. Consequently it is worth sharing the conclusions of our recent Colipa 'Testing Strategies for Skin Sensitisation' workshop (March 2009, Brussels). During this one day meeting, Colipa member companies concluded that the integration of data from different non-animal test methods needs to follow the chemical/biological mode of action that a particular compound or class of compounds will utilize to induce skin sensitization. So for example, data from a test method battery should be integrated and weighted based upon the relative impact that modulation of each key pathway (e.g. peptide reactivity – binding of chemical sensitizer to protein) will have on the overall process of induction of skin sensitisation. Furthermore, such a strategy for test battery integration should be flexible to allow for different test methods that predict the same pathway (e.g. h-CLAT and MUSST) to be used interchangeably and for data generation to be scaled based upon the predicted exposure scenario for a given ingredient (e.g. the requirement for hazard characterization data to support the use of a novel ingredient within a rinse-off, shampoo formulation will potentially vary from those for a leave-on skin cream as the predicted skin exposure will be different). Consequently the ideal solution may be a tiered approach with data from each test method weighted based upon an understanding of the biological/chemical mode of action of the ingredient, which can be flexibly applied based upon the predicted exposure scenario and experience with different test methods. Through applying such an approach to several compounds (and exposure scenarios) it will be possible to define its applicability domain and the remaining gaps (e.g. water insoluble compounds).

To conclude, despite the gaps and challenges that remain, the consensus within Colipa is that our ability to make risk assessment decisions without new animal test data will be possible within the next decade for the majority of ingredients and exposure scenarios.

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