

The COLIPA strategy for the development of *in vitro* alternatives: Skin sensitisation

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Abstract

Allergic contact dermatitis is a delayed-type hypersensitivity reaction induced by small reactive chemicals (haptens). Currently, the sensitizing potential of chemicals is usually identified on the basis of animal studies, such as the local lymph node assay (LLNA) or guinea pig tests. There are, however, increasing public and political concerns regarding the use of animal testing for the screening of new chemicals. The development of *in vitro* models for predicting the sensitizing potential of new chemicals is therefore receiving widespread interest and the COLIPA Project Team Skin Tolerance is involved in a range of research projects exploiting our current understanding of the molecular and cellular events occurring during the acquisition of skin sensitization.

Projects reflecting many aspects of the complex interactions of a chemical with the different compartments of the immune system are being supported: These approaches range from aspects of chemistry/peptide binding/skin metabolism, through evaluation of intracellular signaling pathways induced by allergens, to allergen induced changes in dendritic/Langerhans cells measured at genomic and protein level. Knowledge gained from this research will be used to support the development and pre-validation of novel *in vitro* approaches for the identification and characterization of skin sensitizing chemicals.

Keywords: *in vitro* alternatives, sensitization, dendritic cells, metabolism, haptens

Introduction

Skin sensitization is the induction of an allergic immune response following skin exposure and can be induced by a subset of chemicals. Allergic Contact Dermatitis is the clinical condition that can result from skin sensitization, whose symptoms (e.g. erythema, oedema) are often similar to those of skin irritation. Guinea pig models were historically used to identify whether a chemical had the potential to induce skin sensitization in humans. More recently, a refined, reduced method, the murine local lymph node assay (LLNA) has been employed. Our current aim is the full replacement of the need for animal testing for the skin sensitization endpoint and to this end the COLIPA Project Team (PT-SCAAT) "Skin Tolerance" has undertaken a program of research. These approaches range from developing computer-

based models to predict epidermal bioavailability, to investigating how chemicals are converted within the skin (protein binding, skin metabolism) and characterizing how chemicals activate skin immune cells (investigating dendritic cell intracellular signaling pathways, gene and receptor expression changes (see Fig. 1). Knowledge gained from this research will be used to support the development and pre-validation of novel *in vitro* approaches for the identification and characterization of skin sensitizing chemicals.

Understanding epidermal bioavailability of chemical sensitizers

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,

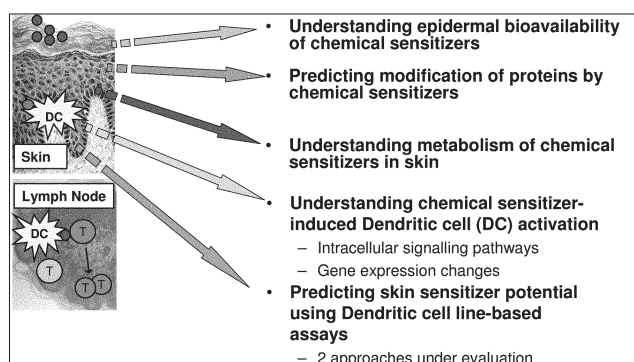


Fig. 1: PT SCAAT Skin Tolerance: Portfolio of Work

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, COLIPA is developing 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 take 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 hours 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.

Predicting modification of 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 chemical (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. 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).

COLIPA is supporting research projects to determine the correlation between sensitization potential and chemical reactivity. In the main project, well-characterized chemical sensitizers and non-sensitizers have been evaluated in a peptide reactivity assay using two synthetic peptides containing either a single cysteine or lysine as a reaction target. After reaction with the test chemical, the samples are analyzed by HPLC to monitor the depletion of unreacted peptides. The data showed that by using a prediction model based on a classification tree approach, peptide reactivity measurement demonstrates a good association between chemical reactivity and allergenic potency (Gerberick et al., 2007). Generally, non-sensitizers and weak sensitizers were found to have minimal to low peptide reactivity, whereas moderate to extremely potent allergens demonstrated moderate to high peptide reactivity.

As a complementary approach, COLIPA has also supported the development of an immunological detection of the cysteine- or lysine-side-chain modification using specific monoclonal antibodies in an ELISA-like format. This immunological approach could provide an alternative and user-friendly system for the detection of protein reactive chemicals. Altogether, these approaches should provide a powerful method for identifying protein reactive chemicals and will be integrated as part of an *in vitro* test battery for identifying skin sensitization potential.

Understanding chemical sensitizer-induced dendritic cell (DC) activation

a) Intracellular signaling pathways

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 and up-regulate the expression of a set of cell surface markers (e.g. CD83, CD86), secrete various cytokines such as IL-1 β 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 an obvious 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. For example, DC maturation following exposure to nickel or DNCB is known to result in an increase of phenotypic marker expression and of inflammatory cytokine secretion.

However, the early intracellular mechanisms involved in DC maturation are not well understood (see Fig. 2). To address this knowledge gap, COLIPA has initiated a research project where DCs derived from human monocytes are treated with sensitizers (nickel, DNCB or thimerosal) in comparison with an irritant (SDS). Upregulation of DC activation markers (CD86, CD54) and cytokine secretion (IL-8 and TNF α) were induced by sensitizers whereas SDS had no effect. Kinase activity measurements (FaceTM 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 pretreatment 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 pretreatment with an antioxidant, N-acetyl-L-cysteine 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 signalling pathways appears to vary between chemical sensitizers (Trompezinski *et al.*, 2007).

b) Gene expression changes

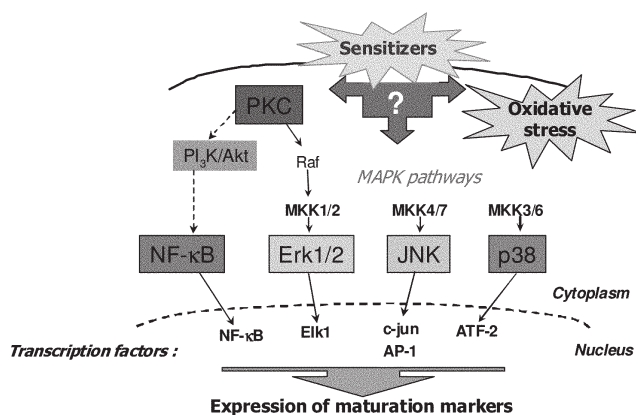


Fig. 2: Intracellular signaling pathways involved in DC maturation

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[®] technology was employed. Two different platforms are being compared: Radix xMAP[®] Bead Assay and Panomics QuantiGene[®] Plex Assay. The evaluation of target genes is continuing and it is hoped that some of the transcript changes identified through this approach will be shown to be suitable for incorporation within a new or existing DC activation assay system.

Understanding metabolism of chemical sensitizers in skin

For the purposes of developing new alternative non-animal testing strategies and to enable us to do mode/mechanism of action based risk assessments relevant to man, it is vital to understand whether or not the envisaged *in vitro* systems are metabolically active and how metabolism of the ingredient *in vitro* compares with that likely to occur in human skin. COLIPA is funding a research program which is aiming to interpret the relevance of *in vitro* data (e.g. from metabolism screens, genetic toxicology assays and skin sensitization cell-based assays), with respect to actual human exposure for cosmetic/toiletry ingredients that are metabolized in the skin.

Future *in vitro* systems could serve to answer two separate metabolism-based questions: The first one is 'What happens to my chemical within skin upon exposure – is it metabolized?' – providing supporting mechanism of action data for use in risk assessment. And the second one is 'Is my *in vitro* tool (for use in a predictive toxicity assay) metabolically competent enough to be able to provide an accurate toxicity endpoint prediction, with respect to actual human skin exposure, for those chemicals whose toxicity is mediated by metabolism? This project is designed with both of these questions in mind. Today, our fundamental understanding of the metabolic competency of human skin is limited (Gibbs *et al.*, 2007). We have begun to extend our knowledge of the metabolic competency of the following models: a) *ex vivo* human skin, b) 3D reconstructed skin models, c) keratinocytes (primary and cell lines) and d) dendritic cells. Functional assays for enzyme activity and proteomics tools are being used to characterize metabolic activity within these model systems.

Predicting DC activation potential using DC-like cell line-based assays

a) DC activation test using the U937 myeloid cell line

Major drawbacks of peripheral blood derived DCs are their complex and expensive preparation procedures and their inherent donor-to-donor variability. As a possible alternative, human myeloid leukemia cell lines represent good candidates as 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. 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 period. The results suggested that a chemical inducing a significant up regulation of the expression of at least two markers might be considered sensitizing. In that first phase, the described test system (U937 activation test) was able to correctly classify 15 out of 16 tested chemicals (Python et al., 2007).

b) human Cell Line Activation Test (h-CLAT)

The h-CLAT was developed using THP-1 cells through a cooperation of two Japanese companies: Kao (H. Sakaguchi) and Shiseido (T. Ashikaga) (Ashikaga et al., 2002). This assay has been evaluated by 5 labs (P&G, Shiseido, Kao, Henkel and L'Oreal) in ongoing collaboration since 2004. The outline of the h-CLAT protocol is as follows: THP-1 cells are treated for 24 h with the test chemical and the expression of cell surface antigens, CD54 and CD86, measured by specific antibody staining and subsequent detection by flow cytometry (See Fig. 3). Two of three independent measurements at any dose should exceed the positive criteria (CD86 \geq 150% or CD54 \geq 200%) in order to be considered as positive. Through the interlaboratory ring trials, some issues (dose setting, prediction model) were identified and the protocol and prediction model have been optimized accordingly. Following the conclusion of the 3rd ring trial, the overall inter-laboratory reproducibility of the test was judged to be good however mis-classifications still exist for a few chemicals, such as hexyl cinnamic aldehyde.

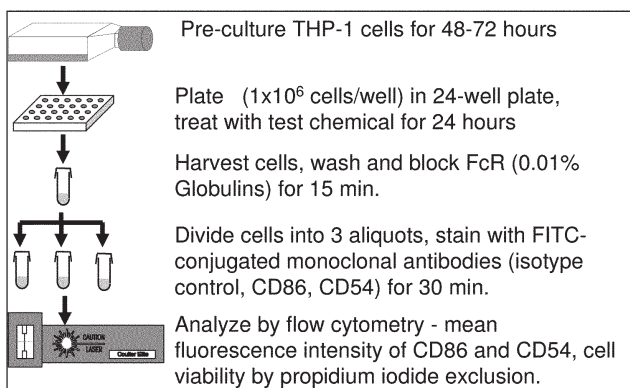


Fig. 3: Outline of the h-CLAT test protocol

c) U937 / CD86 test

The U937/CD86 test protocol was developed by L'Oreal (F. Rousset et al.) and Cosmital SA, Procter & Gamble (F. Python and P. Aeby). For a brief outline, see Fig. 4. In a first phase, it was distributed and evaluated within three laboratories (L'Oreal, Cosmital and LVMH). 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. Overall the conclusion from these activities has been that the U937/CD86 test protocol is promising, transferable and robust.

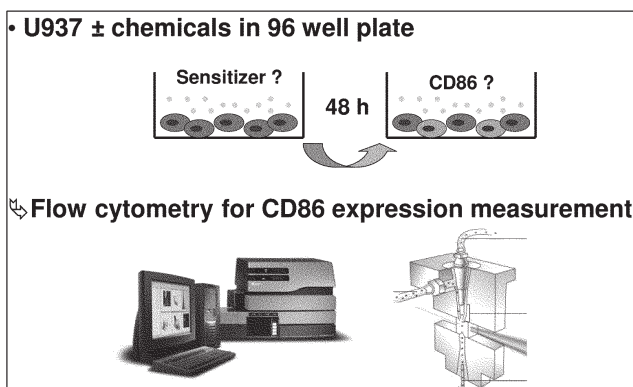


Fig. 4: Outline of the U937 / DC86 test protocol

Collaboration

In our effort to develop *in vitro* skin sensitization approaches, COLIPA is closely collaborating with the research consortium entitled "Novel Testing Strategies for *In Vitro* Assessment of Allergens" (Acronym: Sens-it-iv) sponsored by the European Union Framework Program 6. This project has 28 participating laboratories and its overall goal is the development of *in vitro* alternatives to animal tests for the risk assessment of potential skin and respiratory sensitizers.

What's next: The COLIPA roadmap

COLIPA recently committed to increase the level of funding across all of its Animal Alternatives program (SCAAT) project teams. The Skin Tolerance project team worked to identify knowledge gaps (see below) and is now seeking to establish collaborations in each of these key areas:

- Keratinocyte - DC interactions
- Optimisation of Chemical-induced changes to DC phenotype
- Next Generation Peptide Binding Assay
- Alternative Metabolic System for Peptide Binding Assay
- Mechanistic understanding of Chemical - Cell interactions
- Development of *in vitro* T cell proliferation assay

- Alternative DC activation endpoints for HT screening
- Investigation of predictivity of B cell responses

Overall the strategic goal of this program is to develop a battery of *in silico/in vitro* predictive assays that could be used in concert to identify and potentially quantify the potential of a novel chemical to induce skin sensitization in man. In this way we aim to generate data to inform skin sensitization consumer safety risk assessment decisions in the absence of animal testing.

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- **Gene expression changes:** Procter & Gamble (C. A. Ryan, L. A. Gildea, J. M. Kennedy, L. Foertsch and G. F. Gerberick).

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- **U937 activation test:** Cosmital SA, Procter & Gamble (F. Python, C. Goebel and P. Aeby).

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