Skin Sensitization: Understanding the *In Vivo* Situation for the Development of Reliable *In Vitro* Test Approaches

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Summary
Due to increasing public concern and the adoption of the 7th Amendment to the Cosmetics Directive, the development of in vitro models for predicting the sensitizing potential of chemicals is receiving widespread interest. This overview describes some of our current research projects exploiting known molecular and cellular events occurring during the acquisition of skin sensitization. Once combined in a test battery, these different in vitro approaches are expected to provide reliable methods for the detection of contact allergens.

Keywords: cosmetics, contact allergens, skin sensitizing potential,

Background Information

Allergic contact dermatitis

Allergic contact dermatitis (ACD) 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). There is, however, an increasing public concern regarding the use of animal testing for the screening of new chemicals and further to the adoption of the 7th Amendment to the Cosmetics Directive in Europe, animal testing of ingredients used in cosmetics will be subjected to severe restrictions by 2009 and tightened by 2013. The development of in vitro models for predicting the sensitizing potential of new chemicals is therefore receiving widespread interest.

In vitro sensitization tests need to resume the complex interactions of a chemical with the different compartments of the immune system (Fig. 1): The chemical must penetrate the skin and react with endogenous proteins. Some chemicals, termed prohaptens, require activation through skin metabolism in order to become haptens capable of binding to skin proteins. Haptenated self-proteins are internalized and processed by immature dendritic cells (DC) 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 self-proteins to T-helper cells, resulting in an antigen-specific immune response.

Since the in vitro method for skin penetration is already accepted (OECD guideline 428), our current line of research concentrates on two major aspects of the skin sensitization process amenable to in vitro approaches: protein binding and DC activation. We are thus developing assays for the measurement of the protein binding properties of chemicals and further refining cell based assays for the evaluation of the DC activation potential of chemicals. These two lines of research should provide novel in vitro test systems and endpoints for the development of an in vitro test battery for assessing the sensitization potential of chemicals.

Assays for detecting protein reactive chemicals

A prerequisite for the occurrence of both antibody- and cell-mediated allergic reactions against chemicals is the activation of chemical-specific T lymphocytes. The vast majority of chemical-specific T cells do not recognize the chemical itself but a conjugate of the chemical with a protein fragment (a peptide) presented in class I or class II MHC molecules on the surface of antigen-presenting cells, such as Langerhans' cells. The chemicals can act as an hapten (i.e. it binds covalently to the side chains of amino acids) or as a pro-hapten (which is metabolically or chemically converted to protein-reactive species).

Procter & Gamble, in collaboration with COLIPA (The European Cosmetic Toiletry and Perfumery Association) has therefore initiated research projects to determine the correlation between sensitization potential and chemical reactivity. Chemicals representing allergens and non-allergens have been evaluated in a peptide reactivity assay using two synthetic peptides containing either a single cysteine or lysine as reaction target. After reaction with the test chemical, the samples are analyzed by HPLC to monitor the depletion of unreacted pep-

http://3r-training.tierversuch.ch/en/module_3r/sensitization_in-vitro/acd

tides. 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-allergens and weak allergens demonstrated minimal to low peptide reactivity, whereas moderate to extremely potent allergens displayed moderate to high peptide reactivity.

As a complementary approach (with the support of COLIPA), we are also developing and evaluating an immunological detection of the cysteine- or lysine-side-chain modification using specific monoclonal antibodies in an ELISA-like format. If successful this immunological approach may provide an alternative and user-friendly system for the detection of protein reactive chemicals. Altogether, these approaches, integrated in an *in vitro* test battery for skin sensitization, should provide a rapid, simple and cost effective screening method for new chemicals.

Analysis of the *in vitro* activation of dendritic-like cells (DC)

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 or CD86), secrete various cytokines such as IL-1ß 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.

We have developed and published (Aeby et al., 2004) an *in vitro* test protocol based on human peripheral blood monocytes derived DCs that are exposed for 3 to 30 hours to the test chemicals. DC maturation is evaluated by flow cytometric measurement of the percentage of CD86 positive cells and quantitative measurement of the mRNA expression of interleukin-1ß, interleukin-8 and aquaporin P3 using real time PCR.

This new approach has been used successfully to analyze the sensitizing properties of many chemicals and we are using it for a detailed analysis of the sensitizing properties of p-phenylenediamine (PPD) through its oxidation products and possible inhibition by acetylation in the skin (publication in preparation). We conclude that the described *in vitro* test system allows a refined analysis of the sensitizing properties of chemicals and will further improve product safety.

To identify further DC genes that are modulated by exposure to allergens, the effect of exposure to a contact allergen was also examined at the transcriptional level using Affymetrix GeneChip®. This analysis revealed 173 genes that are significantly modulated (Gildea et al., 2006). It is hoped that some of the identified transcript changes will be suitable for further improving our *in vitro* DC activation assays.

DC activation test using the U937 myeloid cell line

Major drawbacks of PBMDCs are their complex and expensive preparation procedures and their inherent donor-to-donor variability. As a possible alternative, human myeloid leukemia cell

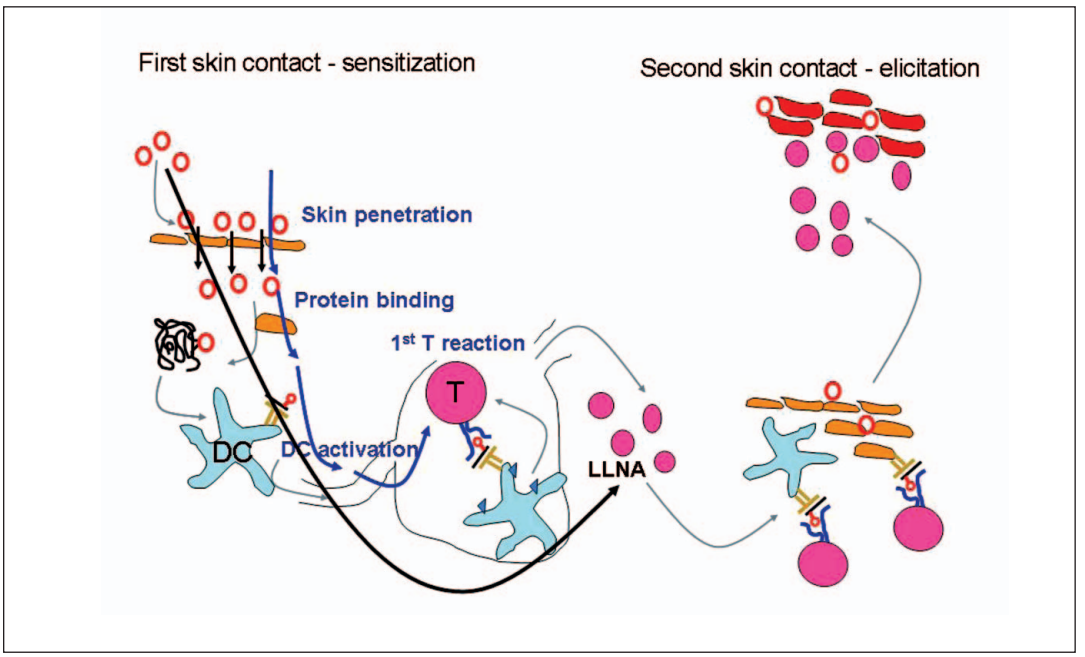

Fig. 1: Interactions of a chemical with the different compartments of the immune system.

lines represent good candidates as DC surrogate. Therefore, in collaboration with COLIPA we have developed and evaluated an *in vitro* test system using the human myeloid cell line U937 for the detection of contact allergens (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-1ß 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-1ß and IL-8) at each time period. Our results suggest 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.

Collaboration with external partners

Due to increasing public and political concerns regarding the use of animal tests for the screening of new chemicals, international co-operations (academic and industrial) are being setup in order to promote and speed up the development of *in vitro* test systems for toxicological endpoints. In our effort to develop an *in vitro* sensitization test, we are closely collaborating with:

A) The European Cosmetic Toiletry and Perfumery Association (COLIPA)

The COLIPA Skin Tolerance Project Team 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.

Research 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. (Aeby et al., 2006)

B) European Union Framework Programme 6 "Sens-it-iv"

We are also involved in a large 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. We, as a Sens-it-iv contractor, are participating in the identification and evaluation of relevant sensitization markers using PBMDCs and the human cell lines MUTZ-3, THP-1 and U937. Our work package involves exposing these cells to known sensitizers under defined culture conditions and total RNA purification for genomic profiling at the Microarray Resource Centre (MARC) of Lund University (part of the Sens-it-iv consortium), using Affimetrix gene arrays. The research is currently progressing and results obtained from the different cellular sources will be compared.

Once validated and combined in a test battery, these different *in vitro* approaches are expected to provide reliable and biologically relevant methods for the detection of contact allergens and will significantly reduce our reliance on animal tests.

References

Aeby, P., Basketter, D., Diembeck, W. et al. (2006). Dendritic cell research projects. *ALTEX. 23, Suppl*., 226-228.

Aeby, P., Wyss, C., Beck, H. et al. (2004). Characterization of the sensitizing potential of chemicals by in vitro analysis of dendritic cell activation and skin penetration. *J. Invest. Dermatol. 122(5)*, 1154-1164.

Casati, S., Aeby, P., Basketter, D. A. et al. (2005). The Report and Recommendations of ECVAM Workshop 51. Dendritic cells as a tool for the predictive identification of skin sensitisation hazard. *ATLA 33,* 47-62, 2005.

Gerberick, G. F., Vassallo, J. D., Foertsch, L. M. et al. (2007). Quantification of Chemical Peptide Reactivity for Screening Contact Allergens: A Classification Tree Model Approach. *Toxicol. Sci. 97*, 417-427.

Gildea, L. A., Ryan, C. A., Foertsch, L. M. et al. (2006). Identification of gene expression changes induced by chemical allergens in dendritic cells: opportunities for skin sensitization testing. *J. Invest. Dermatol. 126(8)*, 1813-1822.

Python, F., Goebel, C. and Aeby, P. (2007). Assessment of the U937 cell line for the detection of contact allergens. *Toxicol Appl Pharmacol. 220(2)*, 113-124.

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