An *In Vitro* Assay to Screen for the Sensitizing Potential of Xenobiotics

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Summary
We are developing a new, animal-free assay for determination of the sensitizing potential of a substance. The design of this assay is based on current immunological knowledge of the pathogenesis of allergic contact dermatitis. It integrates human dendritic cells and keratinocytes, which are both known to be critically involved in vivo. The read-out system uses molecular responses typically occurring after an encounter of the skin with contact allergens. The assay provides concentration-response information, by which the relative ability of a chemical to induce sensitization can be predicted. Additionally, the assay defines the border-concentration of general toxicity of a substance. Weak allergens and even prohaptens are detectable. We have called the assay LCSA, loose-fit coculture-based sensitization assay.

Zusammenfassung: Ein *in vitro* Ansatz zur Prüfung des sensibilisierenden Potentials von Fremdstoffen
Wir entwickeln ein neues, tierversuchsfreies Testverfahren zur Bestimmung des sensibilisierenden Potentials einer Substanz. Der Test basiert auf aktuellen immunbiologischen Kenntnissen über die Entwicklung der allergischen Kontaktdermatitis. Menschliche dendritische Zellen und Keratinozyten, die in vivo maßgeblich an der Sensibilisierung beteiligt sind, werden in das Testverfahren integriert. Als Messgrößen dienen Moleküle, deren Konzentration sich typischerweise nach Hautkontakt mit einem Kontaktallergen erhöht. Der neue Test erstellt eine Konzentrations-Wirkungsbeziehung, auf deren Basis das sensibilisierende Potential einer Substanz ermittelt werden kann. Zusätzlich bestimmt das neue Verfahren die Konzentration einer Substanz, ab der allgemeine Zytotoxizität zu erwarten ist. Schwache Allergene und Prohaptene sind detektierbar. Die Bezeichnung LCSA steht für loose-fit coculture-based sensitization assay.

Keywords: contact allergy, sensitization, in vitro assay, cell culture

1 TeSens

TeSens was founded 2007 in Berlin as a small-sized enterprise. TeSens designs, develops and performs *in vitro* assays (www.tesens.de). We handle a rich variety of cell biological and immunological methods and hold expert knowledge on the biology of Langerhans cells/dendritic cells and keratinocytes. Currently, TeSens is engaged in further evaluation and refinement of the LCSA (Schreiner et al., 2007), a new *in vitro* assay for prediction of the sensitizing potential of a substance.

2 Allergic contact dermatitis / sensitization

Allergic contact dermatitis (ACD) is the most common form of immunotoxicity in humans. In the Central European population, the prevalence of sensitization to at least one contact allergen is estimated to lie between 15 and 20%. Pathogenesis of ACD comprises two phases. During the induction phase, the immune system learns to assess the contact allergen as a dangerous foreign substance. This process is called sensitization. In the elicitation phase, further contact to the sensitizing substance elicits clinical symptoms. Since development of ACD might be prevented by removing sensitizing substances from consumer products, assays for the detection of substances with sensitizing potential have been developed. Currently, the prevalent assay is the guinea pig maximisation assay (Bühler). An alternative is provided by the mouse local lymph node assay (LLNA), which reduces numbers of and burden on test animals. However, it would be ethically desirable to use an animal-free assay. In addition, an acceptable test should be human-specific and should provide dose-response information. Conceivable alternatives may include an intensified implementation of human epidemiological data, like diagnostic patch test data, into hazard identification studies. Prediction might also result from analysis of quantitative structure-activity relationships and their interpolation to new substances. Our approach is *in vitro* testing.

3 The role of keratinocytes and of dendritic cells

To elicit skin contact allergy, a potential allergen first has to adsorb to the skin and needs then to penetrate through the horny layer to reach the viable part of the epidermis (Fig. 1). Potential contact allergens are small molecules that are not immunogenic by themselves. They need to be combined with a protein to

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form a newly shaped molecule, which appears to be foreign to the immune system. Some potential contact allergens are structurally not able to bind to a protein and need to be metabolised to a protein-reactive molecule first. Keratinocytes are involved in both metabolism and proteinisation of potential allergenic molecules (Fig. 1).

In the epidermis, the foreign molecule is taken up by Langerhans cells. These cells belong to the family of dendritic cells (DC). All body regions facing the environment contain DCs. Their role is the detection of invading foreign substances and induction of an appropriate immune-reaction. During evolution, DCs have been equipped with receptors that detect molecules that are typical for pathogenic microorganisms. Contact of these receptors with such danger-signalling molecules activates the DC. It starts to migrate to a lymph node and matures to a professional antigen-presenting cell (APC), which presents parts of the foreign molecule on its surface. In the lymph node, the APC screens for lymphocytes that match the presented antigen and activates them (Fig. 1).

This process of sensitization is required for the induction of a specific immune response. However, sensitization is not restricted to pathogenic microbial molecules. Sensitization to allergenic substances, to molecules of transplanted tissue, or even to the body's own cells may occur (for review see Steinman and Banchereau, 2007; Medzhitov, 2007). Interactions of allergenic substances with DCs are still not well defined. Crucial allergenic properties that determine the immune response are unclear; especially, the nature of the DC activating danger signal is unknown.

Natural DC stem from precursor cells of the bone marrow. In the periphery, two main types of DC can be distinguished: myeloid and plasmacytoid DCs. They differ in body location, in special functions, and in their appropriate protein equipment. The typical myeloid DC of the epidermis is the Langerhans cell. Together with Matthias Peiser, we have established a method to purify Langerhans cells and could describe some of their special features (Peiser et al., 2003; Peiser et al. 2004; Peiser et al., 2008).

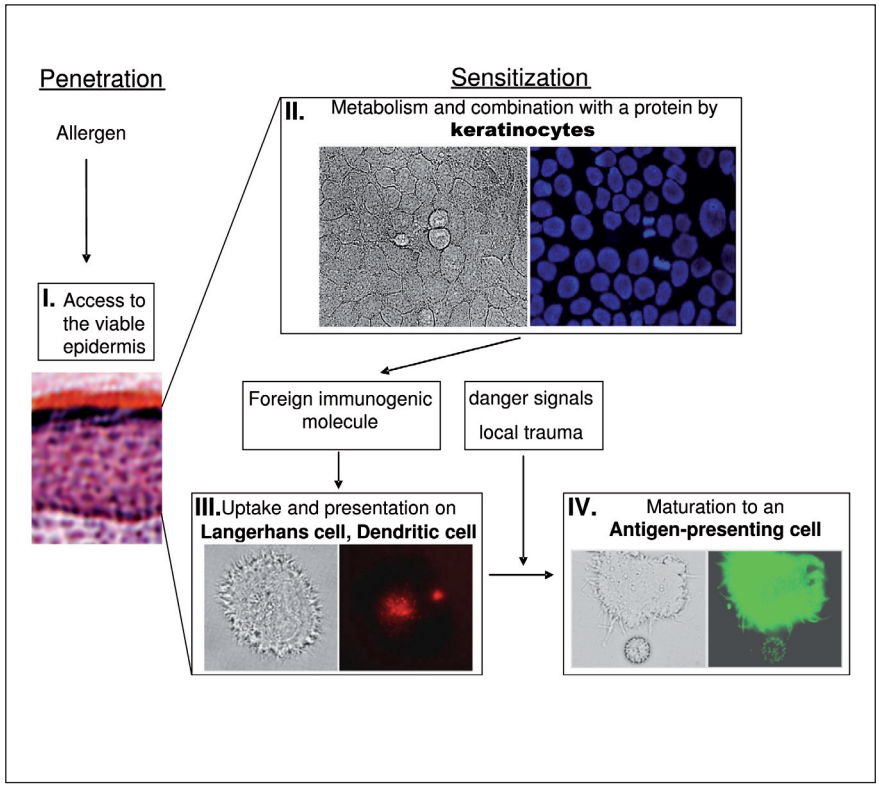

Fig. 1: An *in vitro* sensitization assay should contain a coculture of keratinocytes and dendritic cells

I. *Profile of the epidermis*. II. *Keratinocytes*, on the left, transmission light microscopy of human keratinocytes in cell culture; on the right, fluorescence microscopy after staining of DNA. Note the condensed chromatin in dividing cells. III. *Dendritic cell*, on the left, transmission light microscopy of an immature dendritic cell; on the right, fluorescence microscopy of incorporated CpG oligonucleotides that represent foreign bacterial and viral molecules. IV. *Antigen-presenting cell*, a mature dendritic cell contacts a lymphocyte. On the left, transmission light microscopy; on the right, fluorescence microscopy after incorporation of fluorescent sphingomyelin into plasma membranes.

However, we and others have failed to develop a technique for culturing Langerhans cells for more than 3 days. This precludes their use in bioassays. Subsequently, even in most basic science studies on the biology of DCs, natural DCs are replaced by artificial DC cell types generated *in vitro* from precursor cells. Precursor cells may be monocytes from peripheral blood or $CD34^+$ stem cells cultured in the presence of cytokine cocktails for a couple of days (Sallusto and Lanzavecchia, 1994; Schuler et al., 1995; Steckel et al., 1995; Chapius et al., 1997; Caux et al. 1996; Geissmann et al. 1998).

In conclusion, a cell culture-based assay for sensitization should contain keratinocytes and DCs.

4 Prediction of sensitizing potential by the LCSA

We presume that epidermal Langerhans cells are not constantly fixed to the keratinocytes *in vivo*. They do not wait for invading molecules to come by, but rather screen for them actively. They are already mobile at the immature stage, before they start to migrate to a lymph node. When pieces of epidermis are transferred to tissue culture, the Langerhans cells emigrate into the culture medium. Also, DCs resist a stable integration into 3-dimensional skin models. Therefore, we have emulated physiological conditions of keratinocyte/dendritic cell interactions in the LCSA, the loose-fit coculture-based sensitization assay.

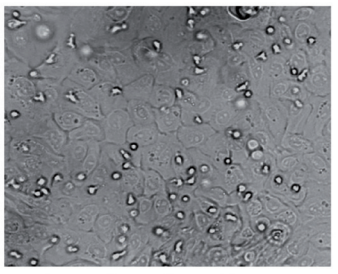

Fig. 2: Loose-fit coculture of human keratinocytes and dendritic cell-related cells

Human keratinocytes and monocytes were cocultured in the presence of a cytokine cocktail. Keratinocytes become activated and monocytes become DC-related cells (DCrc). Such a coculture is ready for application of a putative allergen. Transmission light microscopy. The apparent huge difference in size between the large keratinocytes and the small DCrc above is partially due to the flatness of the keratinocytes. Typical dendritic morphology is less pronounced in DCrc.

The human cells in the assay originate from otherwise discarded material and we use them with the donor's consent.

Human primary keratinocytes are seeded directly onto cell culture plastic material. We do not use a fibroblast layer. Serum-free medium is used for cell culturing. When the keratinocytes have grown to about 50% confluency, allogenic human monocytes are added. Then, cells are cocultured in the presence of a cytokine cocktail including TGF-β. At first, the monocytes adhere to the open areas of the plastic material and to the keratinocytes. After a few hours, they detach and float above the keratinocytes (Fig. 2). In the course of coculturing, both cell types undergo biological modifications. The monocytes differentiate to a cell type that is related to DCs. The typical DC morphology, with prominent dendrites and expression of typical surface protein patterns, is less pronounced compared to natural Langerhans cells or moDCs, which are generated from monocytes monocultured in a medium containing serum. We therefore call the DC-like cells used in the LCSA DCrc, dendritic cell-related cells (Fig. 2). Moreover, the keratinocytes become activated. As known for keratinocytes actively involved in wound healing, they begin to secrete the metalloproteinase MMP-9. The coculture system is now ready for application of test substances.

The first example shows that coculture with keratinocytes increases the sensitivity of dendritic cells. The strong allergen TNBS was applied at a low concentration to either monocytes cultured alone or to monocytes cocultured with keratinocytes under LCSA conditions (Fig. 3A, 3B). Two days after application of the test substance, cells were harvested and stained using antibodies against the myeloid cell marker CD11c and the DC activation marker CD86. In FACS analysis, cells in the DCrc containing region were gated (R1). *In vivo*, activated DCs up-regulate the expression of the co-stimulatory molecule CD86. So, measurement of CD86 provides a suitable read-out system for sensitization. The mean fluorescent intensity of CD86 staining was elevated nearly 3-fold in the LCSA (Fig. 3B), but not in cell cultures missing keratinocytes (Fig. 3A). The dose-response relationship is shown in Figure 4. The LCSA revealed an unprecedented sensitivity to this allergen as compared to other known sensitization assays.

Many contact allergens tend to induce general toxicity when applied at higher doses. Exceeding a concentration limit, they act as irritants. The next example shows test results of the hair-dye PPD (Brandowski's base) in the LCSA (Fig. 5). Here we have included 7-AAD staining in the FACS analysis. Vital cells do not incorporate this substance, so 7-AAD positive staining can be used as an indicator of cell death. Cytotoxicity was evident at PPD concentrations above 25 μM (Fig. 5, broken line). Owing to the high sensitivity of the LCSA, assessment of the sensitizing potential of PPD was possible: CD86 expression

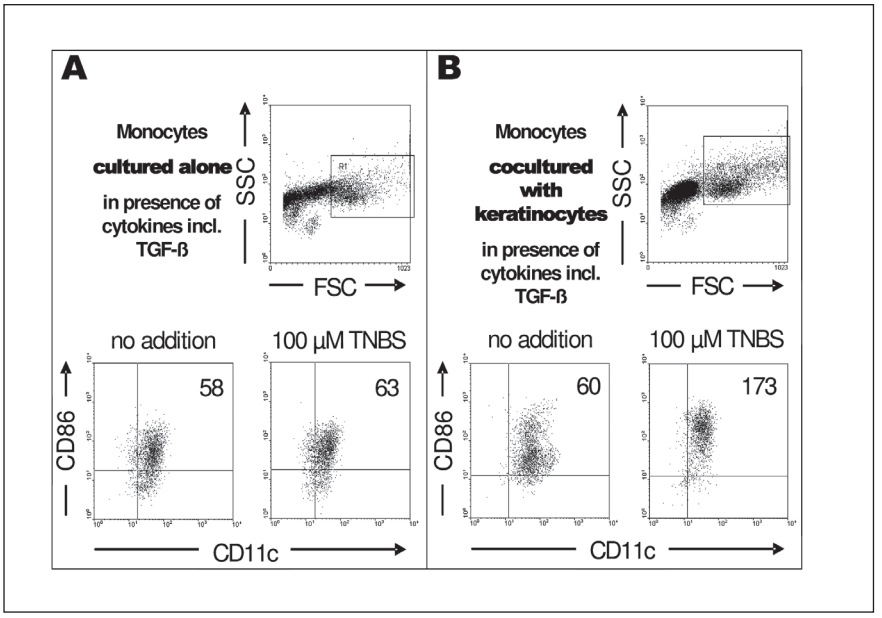

Fig. 3: Coculture with keratinocytes increases the sensitivity of dendritic cells

Dendritic cells were generated from human monocytes either alone (A) or in coculture with keratinocytes (B). No allergen or a low concentration of the strong allergen TNBS were applied. After 2 days cells were analysed by flow cytometry (FACS). Dendritic cells are located in gates R1 of the scatter plots. The protein CD11c marks myeloid cells. CD86 is a costimulatory molecule on dendritic cells. Upregulation indicates dendritic cell activation. Numbers given indicate the mean fluorescence intensity (MFI) of CD86.

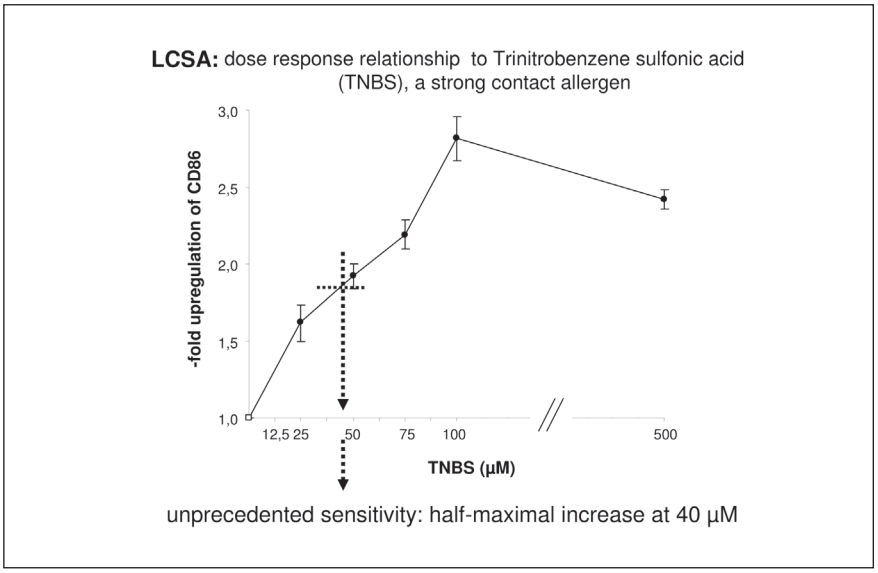

Fig. 4: LCSA, dose-response relationship
Increasing concentrations of the allergen TNBS were applied to LCSA. After 2 days, upregulation of CD86 was determined. On the y-axis, the upregulation of the density of CD86 is shown as MFI CD86 divided by MFI CD86 of the vehicle control.

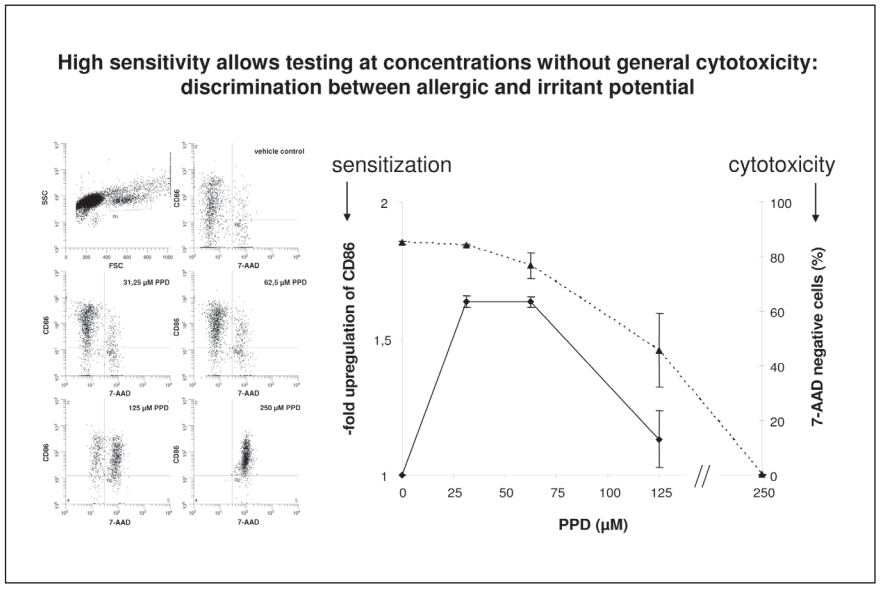

Fig. 5: LCSA, determination of sensitization and cytotoxicity
Increasing concentrations of the hair colorant PPD were applied in the LCSA. After 2 days, the upregulation of CD86 was determined (left y-axis, solid line). In addition, dead cells were stained with 7-AAD. The viable cells are 7-AAD negative (right y-axis, broken line). At a PPD concentration of 250 μM, all of the DCrc were dead. At concentrations above 25 μM, increasing rates of cytotoxicity interfered with determination of sensitization potential.

was already upregulated at 25 μM (Fig. 5, solid line).

SDS, sodiumdodecylsulphate, is an irritant. It does not have sensitizing potential, but is judged a sensitizer in the animal-based LLNA. The LCSA judges it correctly (Fig. 6). SDS at a concentration of 20 μM (which does not yet induce general cytotoxicity) does not cause an upregulation of CD86 expression.

Until now, we have tested 14 different substances (Fig. 7). For each substance, we determine the border to cytotoxicity and compile a dose-response relationship that allows calculating the concentration of half-maximal CD86 upregulation. In addition, we measure secretion of an inflammatory cytokine and a chemokine into the cell culture medium. To classify our data into categories of strength (like strong-moderate-weak), we compare the results with those of known allergens with well established potency.

The LCSA detected the sensitizing potency of the fragrance isoeugenol, which is a prohapten that needs metabolisation to a form that can be combined with a protein. The fragrance α-hexylcinnamaldehyde is a contact allergen that is used as a positive standard in animal-based sensitization assays. For unknown reasons, its potential was hardly detectable in other *in vitro* assays. The LCSA catagorised it as a strong allergen.

When we analyse a substance, we use various pairs of cell donors. As yet, we could not detect a significant cell donor dependent variation of test results. This may partly result from preselection of cells. We include only those batches of monocytes that contain a sufficient fraction of cells undergoing differentiation to DCrc under LCSA cell culture conditions, and we use only keratinocytes with a good proliferation rate. This confers robustness to the assay.

5 Shortcomings of the LCSA as a stand-alone assay

For prediction of the sensitizing potential of a putative allergen that contacts the skin, three main questions need to be answered:

- How much of the substance will penetrate the horny layer?
- Will the substance be metabolised and combined with protein to form an immunogenic molecule?
- Will the sensitizing dendritic cells be induced to mature to antigen-presenting cells?

We do not believe that one single *in vitro* assay will ever be able to give answers to all of these questions. In the sensitization assay, test substances do not need to overcome an obstacle like the horny layer before they reach the target cells. In the LCSA, test substances are added directly to the DCrc. Therefore results of such a sensitization assay need to be combined with data on penetration rates to determine a correction factor for reliable dose-response information. Together with Monika Schäfer-Korting, we are currently combining an *in vitro* assay on absorption and metabolism with the *in vitro* assay for sensitization. In this project, an allergen is placed onto a three-dimensional skin model. During penetration of the substance through the model's cell layers, fractions are gathered and characterised regarding the allergen's concentration and presence of metabolites. These same fractions are used as test substances in the sensitization assay.

Without activation of dendritic cells, the process of sensitization will not be induced. However, dendritic cell activation represents only the initial phase. Sensitization includes priming of T cells. Given that a compound induces dendritic cell activation but does not lead to lymphocyte activation, the LCSA would come to a false-positive result. We are currently working on including T cells in the sensitization assay. The aim is to perform a mixed leukocyte reaction (MLR) using DCrc as drivers of T cell proliferation (combining a non-radioactive type of MLR with dendritic cell activation as described in (Peiser et al., 2007)).

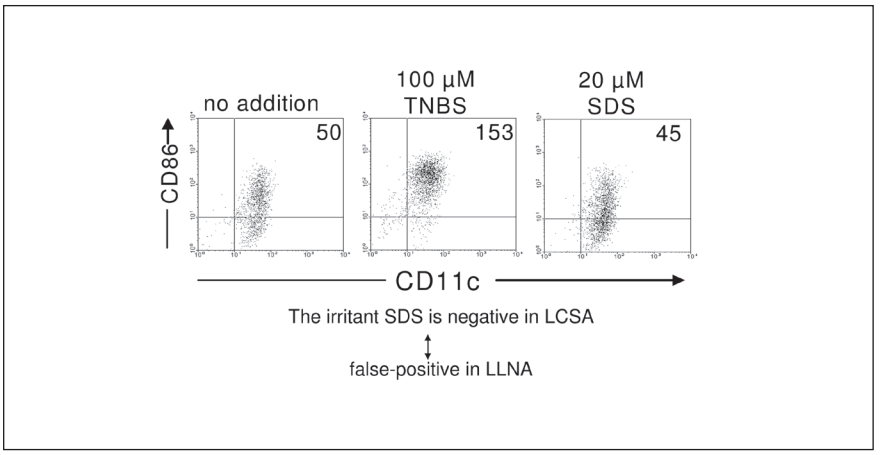

Fig. 6: The irritant SDS is negative in LCSA
Vehicle control (no addition), the allergen TNBS or the irritant SDS were applied in the LCSA. After 2 days, expression of CD11c and CD86 were determined by FACS.

50 different cell-donor pairs	no donor-dependent variance detectable no problem with genetic instability no ethical or logistical problems cells cryopreservable, no use of serum
14 different substances	discrimination between allergens and irritant or between sensitizing and cytotoxic concentration of a substance isoeugenol (prohapten) and hexylcinnamaldehyde (HCA) detectable discrimination between allergens and the pathogenic molecule LPS discrimination between metal-allergens and small-sized molecules

Fig. 7: Performance of the LCSA
Known sensitizing substances tested positive: 2,4,6-trinitrobenzenesulphonic acid (TNBS), PPD (Brandowski's base; itself protein unreactive (White et al., 2006)), the textile dye cleavage products 2-amino-p-cresol and 4-aminoacetanilide (of which only the -cresol revealed sensitizing potential), the prohapten isoeugenol, α-hexylcinnamaldehyde (HCA), cinnamaldehyde, nickel, and cobalt. Irritants tested negative with respect to sensitizing potential: SDS and zink. Solubility vehicles tested negative with respect to sensitizing potential: DMSO and ethanol. Lipopolysaccharide (LPS), a bacterial surface molecule, tested positive for sensitization potential.

6 Conclusion

In a student seminar, the question came up why it is of importance to develop a new assay for sensitization as long as the

cosmetic industry shows no intention to remove well known allergens like the above mentioned substances PPD and HCA from its products. This is a complex issue, and we try to give an answer from the perspective of an assay developer. The numbers of sensitizing substances in today's consumer products are fairly high. It does not seem feasible to remove all of them completely. More practical would be a reduction to harmless concentration ranges. The acceptable maximal concentration of a given sensitizer must be defined. To become widely accepted, the determination of tolerable amounts of an allergen in an end product must rely on a sharp, quantitative assay. Animal assays do not meet such a requirement. The LCSA can provide dose-response information.

Development of the LCSA has been promising. It proved sensitive enough to detect even weak allergens and prohaptens. Now it must be assessed whether it is robust enough for routine screening.

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