

DNA microarrays provide new options for allergen testing

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Sandra Szameit[†],
Elisabeth Weber and
Christa Noehammer

[†]Author for correspondence
Toxicology, Seibersdorf Labor
GmbH, A-2444 Seibersdorf,
Austria
Tel.: +43 505 503 546
Fax: +43 505 503 653
sandra.szameit@seibersdorf-laboratories.at

Microarray studies are increasingly used for toxicological research and even for the development of new toxicological test methods. Since gene-expression changes in cultured cells can be conveniently measured with microarrays, this method might be of use for *in vitro* toxicity testing, for example, in the field of contact sensitization. Allergic contact dermatitis, the clinical manifestation of contact sensitization, may occur when sensitizing chemicals enter the skin and get in contact with epidermal and dermal antigen-presenting cells. The resulting maturation process in these cells can be measured by employing gene-expression analysis. Biomarkers currently known seem to be insufficient to identify all kinds of contact sensitizers, which may partly activate different signaling pathways (e.g., metal or organic sensitizers). Therefore, genome-wide screenings using whole-genome DNA microarrays and extensive data analysis can be performed in order to identify additional genes. Ultimately, marker genes detected in whole-genome experiments can be included in small-scale-targeted microarrays in order to establish the final test method.

KEYWORDS: antigen-presenting cell • biomarker • contact sensitization • dendritic cell • *in vitro* • microarray • toxicology testing

Contact sensitization & toxicology testing

Allergies may affect different parts of the body, such as the airways, nose and sinuses, eyes, ears, GI tract or the skin [1]. Out of these, contact sensitization causing dermal allergy is one important issue, since allergic contact dermatitis (ACD) is one of the most common skin diseases. Clinically, ACD is characterized by redness, papules and vesicles, followed by scaling and dry skin [2]. The skin lesions are concentrated on the site of allergen contact; however, they may propagate in the immediate vicinity or to distant unrelated sites.

The sensitization process resulting in ACD is T-cell mediated and occurs in two distinct phases (FIGURE 1). The first phase is the 'sensitization phase' and develops during the first immunologically relevant contact of the allergen with the skin. The allergen (usually low-molecular-weight haptens < 500 Da) penetrates into the viable layers of the epidermis, binds to carrier proteins and comes into contact with immature Langerhans cells (LCs), the antigen-presenting cells (APCs) of the skin. The innate immunity system of the skin is activated by proinflammatory properties of the chemicals, and migration and maturation of LCs is induced [3]. The hapten–protein complexes are then taken up

and processed by LCs and are expressed as haptenated peptides by the major histocompatibility complex at the cell surface. In this way, the LCs are activated and migrate to the draining lymph nodes where they present the antigen to T lymphocytes. Thus, specific T lymphocytes expand clonally, differentiate into effector memory cells and central memory cells, and diffuse to the bloodstream through the efferent lymphatics [4].

Only during the second phase of ACD, a clinically visible reaction can be seen. This 'elicitation phase' occurs after a challenge of sensitized individuals with the same allergen. After penetrating the skin once again, the hapten is presented to specific effector and memory T lymphocytes by different APCs (e.g., macrophages or B lymphocytes). As a consequence of this, the T lymphocytes are activated to trigger the inflammatory process through cytokine/chemokine production and keratinocyte apoptosis, clinically visible as edema in between the keratinocytes (spongiosis). Finally, the recruitment of leukocytes from the blood to the skin amplifies the development of skin lesions [3].

Individuals affected by ACD are exposed to great physical and mental suffering. While identification of the cause of ACD is generally possible by patch testing, the only available treatment

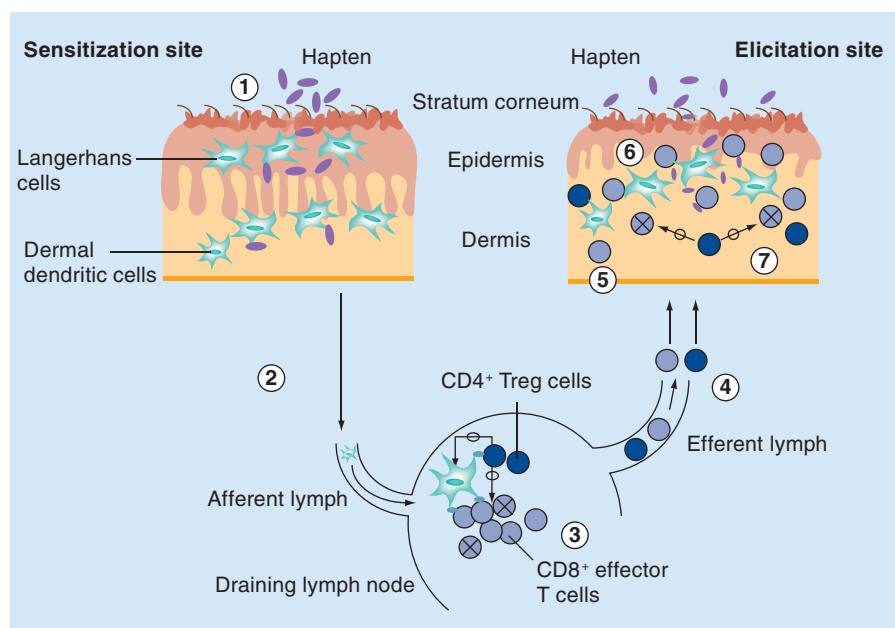


Figure 1. Mechanism of allergic contact dermatitis. Sensitization step: Haptens penetrate the horny layer of the skin (stratum corneum). (1) Hapten loading by skin dendritic cells (DCs) parallels activation and (2) migration of DCs through the afferent lymphatic vessels to the draining lymph node (LN). Migrating DCs are located in the para-cortical area of the draining LN where (3) they can present haptenated peptides on major histocompatibility complex class I and II molecules to CD8⁺ effector T cells and CD4⁺ Treg cells, respectively. (4) Specific T-cell precursors expand clonally in the draining LN and diffuse to the bloodstream through the efferent lymphatic vessels and the thoracic duct. During this process, they become memory T cells. Primed T cells preferentially diffuse in the skin after transendothelial migration. At the end of the sensitization step, everything is ready for the development of a visible reaction upon challenge with the relevant hapten. Elicitation phase: (5) when the hapten is painted again, it diffuses through the epidermis and is loaded by Langerhans cells or other skin cells expressing major histocompatibility complex molecules, such as keratinocytes and dermal DCs, which are then able to activate trafficking-specific T cells. (6) CD8⁺ cytotoxic T-cell activation initiates the inflammatory process through keratinocyte apoptosis and cytokine/chemokine production. (7) This is responsible for the recruitment of leukocytes (including Treg cells) from the blood to the skin leading to the development of skin lesions. Reproduced with permission from [3].

of the allergy is elimination or avoidance of the contact allergen. Therefore, it is crucial to analyze chemicals on the market and classify sensitizing substances correctly. Testing of chemicals for potential sensitizing effects is currently performed using animal tests, such as the guinea pig maximization test [5] or the local lymph node assay (LLNA) [6]. However, owing to current legislation, the development of *in vitro* assays as alternatives to animal tests for toxicological issues has become increasingly important.

Several key elements of the sensitization process are considered eligible for the development of nonanimal-test methods, namely ability of the chemical to cross the skin barrier, binding of the chemical to proteins, uptake of the chemical by APCs, maturation and migration of the APCs, and finally activation of T lymphocytes (reviewed in [101]). Cell-based assays employing cell types that take part in the sensitization process *in vivo* are being developed, using keratinocytes [7], dendritic cells (DCs), cocultures with keratinocytes and DCs [8], or T-lymphocyte priming assays [9,10].

This article covers the important aspect of maturation of APCs during hapten uptake and presentation. Basically, exposure of the cells to sensitizers induces the maturation process, while treatment with irritants does not stimulate maturation. The maturation process is characterized by morphological and functional changes in APCs. While immature cells are situated in the skin and act as surveillants against foreign antigens, mature cells migrate to the draining lymph nodes in order to present the antigens to T lymphocytes. These changes imply alterations in gene expression, which can be used to discriminate immature and mature cells. Several groups work on the establishment of *in vitro* assays using either primary cells derived from human blood or skin or different cell lines.

Tests based on cell lines

The usage of cell lines has several advantages, such as availability and abundance of the cells. Furthermore, variability between different cultures of the same cell line should be low. Two Japanese companies developed the human cell line activation test that is performed with THP-1 cells, a human monocytic cell line. The test is based on flow cytometric measurement of the maturation markers CD86 and CD54 [11,12]. Other groups also investigated the usability of THP-1 cells [13]. They published IL-8 as a potential discriminative marker for sensitizers and irritants. In addition, they showed the activation of the p38 MAPK pathway after exposure of the THP-1 cells to allergens [13]. A comparison

of CD86 and CD54 cell surface expression on THP-1 cells and primary DCs cultured from blood showed comparable results, again indicating the usability of THP-1 cells for the development of an *in vitro* test system [14]. The human myeloid cell line, U937, was also taken into consideration for the human cell line activation test [11,12], and the U937 activation test was presented in 2007 by Phyton *et al.* [15]. A combination of selected markers, namely surface protein expression of CD86, and gene expression of IL1- β and IL-8, was proposed for this assay. Furthermore, the acute myeloid leukemia-derived cell line, MUTZ-3, was used as model system for primary monocytic cells already in 1996 [16,17]. Later, the capability of MUTZ-3 cells to acquire a DC-like phenotype and T-cell stimulatory capacity was stated [18,19]. Larsson and colleagues extensively studied the capability of the cell lines KG-1, THP-1 and MUTZ-3 to differentiate into functional DCs and could show significant similarities between MUTZ-3-derived DCs and primary DCs cultured from human blood. The

similarities cover expression of surface markers (e.g., D1a, CD14, CD86, CD80 and HLA-DR), capacity of the cells to stimulate T lymphocytes and gene-expression profiles [20]. Flow cytometric analysis of MUTZ-3 cells exposed to various sensitizers and irritants indicated the usability of CD86 as marker for contact sensitization [21].

Tests based on DCs/LCs

While cell lines as model systems for DCs obviously have advantages, we found that, compared with primary DCs, protein expression of the maturation marker CD86 was significantly lower in U937 and MUTZ-3 cells, indicating a lower degree of maturation [SZAMEIT *ET AL.*; UNPUBLISHED DATA]. We concluded that cell lines might not be the best choice for all applications, such as for gene-expression analysis.

Since LCs are the primary APCs of the skin, experiments have been performed that aimed to employ LCs for the establishment of *in vitro* assays [22,23]. However, these cells constitute only 1–3% of all epidermal cells and, thus, are difficult to isolate in sufficient numbers. Furthermore, they very often mature during the isolation procedure [23].

Owing to the challenges concerning the use of LCs, currently, two different types of DC-like cells are under investigation. In 1994, it was shown that DCs can be cultured *in vitro* from human blood monocytes [24,25]. Another option is the cultivation of DCs derived from CD34⁺ stem cells from cord blood [23,26,27]. Both cell types are currently employed for the development of *in vitro* tests for contact sensitization. Early works concentrated on the expression analysis of surface markers (e.g., CD86, CD83, CD80, CD54 and HLA-DR), and certain cytokines, especially IL1- β [23,28–35]. Over the years, functional studies of the sensitization process led to the identification of more relevant genes, such as *CCR7* [36,37] and *IL8* [38]. However, identification of weaker sensitizers is still challenging and can be easier with additional markers. Gene expression analysis of chemical-treated DCs might result in the detection of new genes differentially expressed after allergen exposure.

Microarray-based *in vitro* test systems

The high-throughput technology of DNA microarray analysis provides a convenient possibility for gene-expression analysis for toxicological issues.

The development of RNA amplification and signal amplification protocols for microarray applications facilitates the analysis of limited starting material (e.g., primary cells). This also helps to analyze changes in transcription during the maturation process of DCs, which was already performed by some groups [39–44]. While genome-wide screening with DNA microarrays is well suited to gaining new insights into DC biology in general, this technique might also result in new marker genes for the identification of contact sensitizers.

In previous works, we established the immune toxicity chip, an immune-specific DNA microarray containing probes for genes, which had already been shown to be involved in DC maturation or antigen-presenting processes [45]. Discrimination of strong allergens and irritants was possible with this microarray; however,

weak sensitizers were not recognized. In order to improve our test system, we performed a whole-genome analysis of DCs exposed to three allergens (NiSO₄, Bandrowski's base and dinitrobenzenesulfonic acid) and two irritants (sodium dodecyl sulfate and TritonTM X-100) and detected a large number of genes that were differentially expressed after allergen but not after irritant exposure (1741 genes upregulated, 2007 genes downregulated) [46]. While these results, and results from similar studies, already indicate that new relevant marker genes can be found, long gene lists alone will not result in an applicable *in vitro* test [47,48]. Therefore, we further analyzed our data, aiming at establishing a complete test assay, including data evaluation and classification of chemicals. Gene selection was performed employing a nearest shrunken centroid method [49] in order to find a combination of genes that can be used in a pattern-recognition approach to determine the sensitizing potential of chemicals. As a result, six genes were found, which are sufficient to discriminate contact allergens and irritants, if the analysis is based on the dataset generated during our work.

More chemicals have to be tested in order to verify this classifier of six genes; however, a high prediction accuracy (100% with the five chemicals tested) and the low number of genes are very encouraging. While the identification of the genes of the classifier was performed with whole-genome microarrays, routine analysis could be performed with a targeted microarray or real-time PCR containing the essential genes only. Therefore, setting up an enlarged immune toxicity chip with probes for the genes of the classifier might allow identification of a broader spectrum of sensitizers.

The Flemish Institute for Technological Research (VITO) in Belgium also started with a genome-wide analysis of gene expression patterns in DCs exposed to contact allergens and irritants [47]. The group then selected the most promising candidate genes and analyzed a larger panel of chemicals with real-time PCR [50]. By constructing a predictive classifier with a transcriptional reference dataset of 21 chemicals, they established the VITOSENS[®] model, which is another example of a sophisticated analysis of gene-expression data leading to applicable test methods.

Over the last few years, other groups also explored gene-expression changes in DCs exposed to contact sensitizers or irritants. Cluzel-Tailhardat and colleagues used a low-density microarray containing probes for 165 genes related to DC biology. Testing four sensitizers and one irritant, they identified specific gene-expression patterns for each chemical employing principal component analysis [51]. The fact that no common gene signature could be found for all allergens indicates that whole-genome microarray analysis might be the way forward in order to find a panel of genes that are up- or down-regulated after exposure to each class of sensitizing chemical. The first genomic-scale microarray analysis in allergen-treated DCs was described by Ryan *et al.* [48]. While only one allergen (dinitrobenzenesulfonic acid) was analyzed in this work, the group published a continuation in 2006, describing real-time PCR results from DCs exposed to a broader spectrum of chemicals [52]. Candidate genes for the real-time PCR were selected from the first work. In total, 11 genes were found that

appeared to have the most predictive potential. These examples demonstrate that the basic strategy is to start with genome-wide expression analysis and then to narrow down the analysis to the most interesting genes, which is widespread and fairly promising.

Expert commentary

Targeted microarrays in combination with a suitable cell system as tools for the discrimination of contact sensitizers and irritants present a promising approach for an *in vitro* test method. Compared with other expression profiling methods, small-scale microarrays present suitable high-throughput systems for the screening of chemicals. On the one hand, compared with whole-genome microarrays, small-scale arrays are considerably cheaper, easier to handle and analyze. On the other hand, compared with

real-time PCR assays, more genes can be analyzed simultaneously and at lower costs. Several hundred marker genes, for example, for contact sensitization, respiratory sensitization and also irritation, can be included on one microarray and tested simultaneously.

In addition, the analysis of a broader panel of marker genes enhances the sensitivity of the DC-based test assay. Many marker genes known until today are also detected after irritant exposure, although lower expressed, so that additional markers would be advantageous.

As an example, FIGURE 2 compares the percentage of CD86⁺ and HLA-DR⁺ cells after treatment of the cells with different sensitizers (NiSO₄ and Bandrowski's base) and one irritant (sodium dodecyl sulfate). While NiSO₄ can easily be classified as a sensitizer based on these two markers (80.84% CD86⁺ cells and 74.23% HLA-DR⁺

cells), the discrimination of the sensitizer Bandrowski's base (49.50% CD86⁺ cells and 36.98% HLA-DR⁺ cells) and the irritant sodium dodecyl sulfate (24.63% CD86⁺ cells and 24.65% HLA-DR⁺ cells) is more difficult in this system.

However, some obstacles still have to be met. First, chemicals insoluble in water might be a challenge to apply to a cell culture system. While it is possible to dissolve the chemical in another solvent, (e.g., dimethyl sulfoxide), the final solvent concentration must not induce toxicity in the cells. In this sense, it might be interesting to additionally use the microarray technique for systems not dependent on water solubility of the chemicals (e.g., artificial skin models). Until now, commercially available skin models (e.g., EpiDermTM, EST-1000TM, AST-2000, EpiSkin[®] and Phenion[®] Full Thickness Skin model) do not include immature DCs. While promising research is ongoing in this field [53], application of a coculture system consisting of a skin model and immature DCs might also be interesting.

Second, as shown in FIGURE 2, the percentage of cells of a chemical-treated culture that take part in the maturation process and the final maturation status of the cells differs between contact sensitizers with varying sensitizing potential.

Therefore, DCs that are exposed to a chemical, and then used for RNA extraction and gene-expression analysis, consist of different amounts of mature and immature DCs. Expression of marker genes for contact sensitization will change during the maturation process, so that the RNA of markers will be present in either mature or immature DCs but not in all cells of the culture. Consequently, changes in RNA amounts of

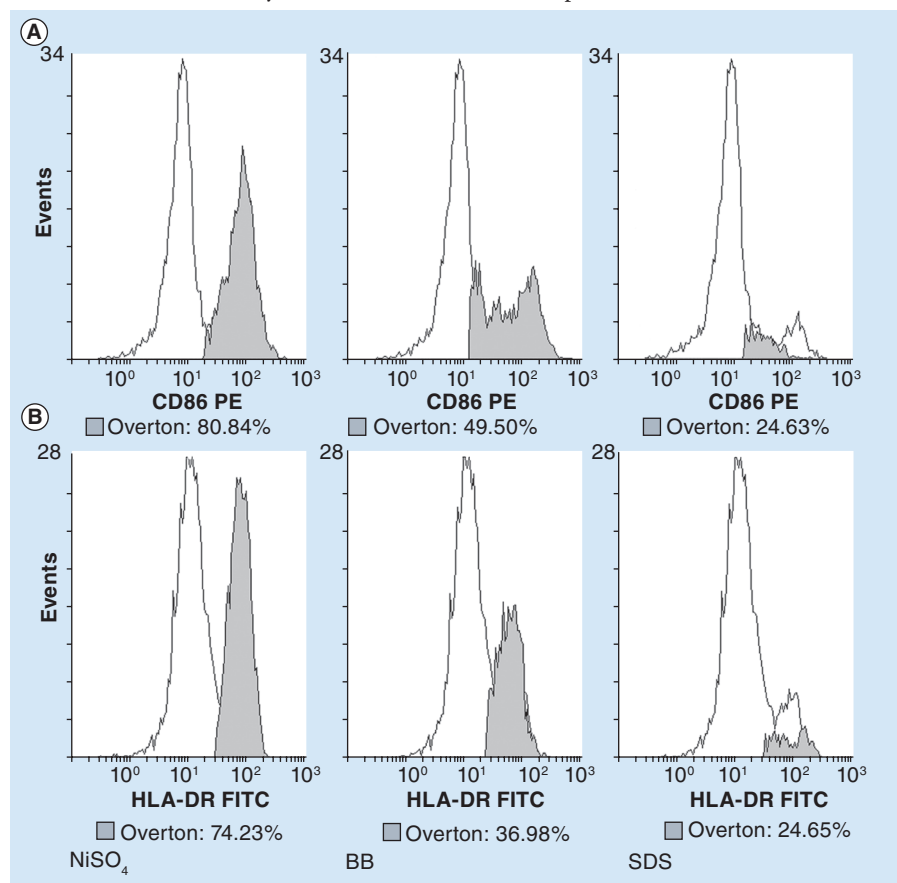


Figure 2. Maturation effects in dendritic cells (DCs) exposed to contact sensitizers and irritants.

DCs cultured from human blood monocytes were treated with the contact sensitizers NiSO₄ and BB, and with the irritant SDS, or with the corresponding solvent control, for 24 h. Nontoxic concentrations of the chemicals were chosen (NiSO₄: 300 μmol/l; BB: 3 mg/l; SDS: 10 mg/l). Cells were analyzed for expression of the maturation markers CD86 and HLA-DR by employing flow cytometry. To calculate the percentage of cells positive for the marker (dark histograms), histogram subtraction was performed after the method of Overton, as previously described [59]. The fluorescence histogram of solvent-treated DCs (light histograms) was taken as a control. Exposure of the cells to NiSO₄ resulted in 80.84% CD86⁺ cells and 72.23% HLA-DR⁺ cells, while exposure of the cells to BB resulted in a lower percentage of mature DCs, with 49.50% CD86⁺ cells and 36.98% HLA-DR⁺ cells. After irritant treatment, mature DCs were also detected, but to a lower degree (24.63% CD86⁺ cells and 24.65% HLA-DR⁺ cells). BB: Bandrowski's base; SDS: Sodium dodecyl sulfate. Reproduced with permission from [45].

the marker genes will be diluted. Care has to be taken to find a chemical concentration that allows the determination of differences in RNA amounts between allergen- and irritant-treated cells most clearly.

Last, owing to the huge amount of data, interpretation of microarray experiments is challenging, especially if whole-genome experiments are performed. A profound data analysis is necessary in order to obtain biologically correct and statistically significant results. Ultimately, an *in vitro* test system based on a targeted microarray should, therefore, include an easy-to-use analysis software that can be applied in every toxicological testing facility.

Five-year view

The development of *in vitro* tests for toxicological testing is a crucial issue, particularly for the cosmetics industry. The 7th Amendment of the European Cosmetics Directive was postulated in 2003, imposing deadlines for banning animal testing, not only for cosmetic products themselves but also for their ingredients [102]. Both a testing ban and a marketing ban are established in the directive. The testing ban prohibits the testing of finished cosmetic products or of cosmetic ingredients on animals. The marketing ban does not allow the marketing of finished cosmetic products or of cosmetic ingredients which were tested on animals within the EU. Different deadlines that will apply stepwise as soon as validated alternatives exist were introduced for the realization of the bans. Finally, the last deadline in March 2013 places a complete marketing ban irrespective of the availability of nonanimal tests [103].

Analysis of changes in the expression of certain genes in maturing DCs is one promising approach for the development of an *in vitro* test for contact sensitization. Since a larger panel of marker genes might be needed to categorize chemicals correctly, a high-throughput method allowing the parallel analysis of many genes might be favorable.

Already in 2002, research in allergy, especially in the identification and characterization of sensitizing substances, was predicted to profit significantly from the microarray technology [54]. While in the beginning of the microarray era, most laboratories were not equipped for the costly new technology, the method is now widespread and also contract research organizations for toxicological testing often have the opportunity to use it. The development of small-scale microarrays by some laboratories [45,54,55] and companies (e.g., Sabiosciences, ArrayIt and Applied Microarrays, Inc.) makes the technology cheaper and analysis easier. Based on this development, employment of the microarray technology for toxicological issues will probably increase further.

In addition, the detection of genes involved in contact sensitization allows analysis of the biological functions of these genes, which should reveal insights into the biology of skin sensitization. Understanding the pathways occurring during the sensitization process is also one of the goals of the Sens-it-iv project, a Sixth Framework research program that aims at developing *in vitro* tests for the identification of skin and respiratory sensitizers [56].

Owing to large interlaboratory projects, such as Sens-it-iv, good progress has been made concerning the development of *in vitro* methods. As an example, the European Cosmetics Association

Colipa coordinates and supports projects covering different aspects of skin sensitization [57]. The European Centre for the Validation of Alternative Methods has successfully coordinated the validation process for many nonanimal methods and is also very active in the field of sensitization testing (e.g., within the Sens-it-iv project).

Well-coordinated cooperation between laboratories will be essential for the validation of *in vitro* assays. While small-scale microarrays are very well suited for a high-throughput screening of chemicals at reasonable costs, cultivation of DC-like primary cells is expensive and the amount of cells is often limited, since they have to be cultured from human blood. Therefore, the amount of substances that can be analyzed by one laboratory is often rather low; however, within interlaboratory projects, the collection of enough reliable data for validation can be performed.

With regard to the different key elements of contact sensitization (see 'Contact sensitization and toxicology testing'), the question arises whether an *in vitro* method that focuses on a single step of this process can be sufficient as replacement of the *in vivo* methods (e.g., the LLNA). During a LLNA, a test substance is applied to the ears of mice and finally proliferation of the cells of the lymph nodes is measured. Therefore, all important steps of the sensitization process have to take place in order to get a positive LLNA. By contrast, a chemical that gives a positive response with maturing DCs might not be able to cross the skin barrier. Consequently, the animal-free test method will not be able to provide a holistic picture of all steps that could induce skin sensitization. Jowsey and colleagues discussed this issue in 2006 and proposed a tiered-testing approach based on the combined information derived from different *in vitro* tests covering all key elements of contact sensitization [58]. Assuming that, ultimately, an overall picture can only be created by looking at different details, most probably different *in vitro* (and also *in silico*) test methods have to be validated until skin sensitization can be tested exclusively with nonanimal methods.

Financial & competing interests disclosure

The authors are named inventors on a related patent application. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Key issues

- Low-molecular-weight chemicals can induce the inflammatory skin disease allergic contact dermatitis.
- Maturation of antigen-presenting cells of the skin is a key element of the sensitization process.
- Current toxicology research aims at developing surrogate *in vitro* tests.
- Microarray technology can be used to search for sensitization-specific biomarkers.
- Small-density microarrays targeting only the relevant marker genes can be integrated in testing platforms for skin sensitization.

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Affiliations

- Sandra Szameit
Toxicology, Seibersdorf Labor GmbH, A-2444 Seibersdorf, Austria
Tel.: +43 505 503 546
Fax: +43 505 503 653
sandra.szameit@arcs.ac.at
- Elisabeth Weber
Toxicology, Seibersdorf Labor GmbH, A-2444 Seibersdorf, Austria
- Christa Noehammer
Molecular Medicine Unit, Health and Research Department, Austrian Institute of Technology, A-2444 Seibersdorf, Austria