

Chemical Sensitization and Allergotoxicology

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Abstract Chemical sensitization remains an important environmental and occupational health issue. A wide range of substances have been shown to possess the ability to induce skin sensitization or respiratory sensitization. As a consequence, there is a need to have appropriate methods to identify sensitizing agents. Although a considerable investment has been made in exploring opportunities to develop methods for hazard identification and characterization, there are, as yet, no validated nonanimal methods available. A state of the art of the different *in vitro* approaches to identify contact and respiratory capacity of chemicals is covered in this chapter.

Keywords Gene biomarkers • *In chemico* models • *In vitro* models • *In silico* models • Mechanistic understanding • Potency • Respiratory sensitization • Sensitization • Skin sensitization

Introduction

Chemical-induced sensitization is the induction of the allergic immune response following exposure to a chemical. Allergic reactions to chemicals predominantly manifest adverse effects in the skin and the respiratory tract. Skin sensitization results in allergic contact dermatitis, and respiratory sensitization causes allergic

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rhinitis and asthma. Following topical exposure, contact sensitization is the usual outcome. The skin is also the target organ following oral exposure. A major route of exposure that is of concern for respiratory allergies is inhalation, but the skin and the gastrointestinal route may also be of importance.

Allergotoxicology studies the influence of pollutants on the development of allergic reactions and diseases [1]. Interaction between air pollutants and allergens can take place both outside and inside the exposed person and, as a consequence, influence allergic diseases. Pollutants may be the carriers of allergens and may exacerbate allergic reactions and diseases [2].

The health problems of allergic diseases together with the continuous introduction of new chemicals to workers and consumers make the identification of agents with a high likelihood to induce allergic sensitization a challenging area in immunotoxicology. The European REACH (Registration, Evaluation, Authorization and restriction of Chemicals) legislation [3] and the 7th Amendment to the Cosmetics Directive banning *in vivo* testing for cosmetics products and their ingredients [4] emphasize the need for nonanimal alternative test methods and testing strategies. Moreover, the vision of toxicity testing in the twenty-first century of the National Research Council (NRC) [5] describes a paradigm shift in toxicity testing and risk assessment that focuses on “toxicity pathways.” The goal is to determine how exposure to environmental/occupational agents can perturb molecular pathways that maintain cell function, causing a cascade of subsequent events leading to adverse health effects. The envisaged process includes chemical characterization, target-specific and mechanism-based toxicity testing, and dose–response and extrapolation modeling based on population-based and human exposure data for translating cellular tests to whole human systems. Where animal experiments used to be the most important approach for toxicity testing, the future is seen in the strength of quantitative high-throughput *in vitro* and *in silico* approaches based on human material and advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology (e.g., (Q)SAR) [6].

Nonanimal Approaches for Sensitization Testing

Non-Cell-Based Approaches to Assess Sensitization

In line with applicable legislations and the above-described altered toxicology vision, considerable efforts are being performed to accurately identify and characterize skin and/or respiratory sensitizing chemicals without the need for animal experimentation. One can identify three main strategies (*in silico*–*in chemico*–*in vitro*) that each show limitations and inconsistencies, making them not suitable for use as single predictors for sensitizing properties [7–9]. Since sensitization is a multistage toxicological process, each of these strategies may

represent different stages and/or chemical classes and be ultimately combined in an integrated approach.

For the identification of skin and respiratory allergens, the use of *in silico* approaches has made its entrance in the last decade. These approaches identify allergens using (quantitative) structure activity relationships ((Q)SAR) methods, which involve a combination of computational chemistry, biology, and statistics [10–13]. (Q)SAR have been used to correlate chemical function with chemical structure, on the assumption that chemical structure is a determinant of reactive properties and hence biological activity. *In silico* models should be built on a good set of empirical data of adequate chemical coverage. The approach to estimate the skin sensitization potential of a chemical *in silico* is based on the concept that the ability of chemicals (haptens) to react with proteins to form covalently linked conjugates, based on electrophilic characteristics in their structure, is correlated with their skin sensitization capability [14]. For skin sensitization, the available published QSAR models fall into either a mechanism-based category or are empirically derived using statistical approaches (global models). Where the former is developed for small sets of structurally similar chemicals [15], the latter is often based on large datasets of diverse structures without an attempt to rationalize the underlying sensitization mechanism [16]. Because of the uncertainties regarding the molecular mechanisms for low molecular weight (LMW) respiratory sensitizers, (Q)SAR seem particularly appropriate because they make no *a priori* mechanistic assumptions [17].

Additionally, non-cell-based *in chemico* approaches are being explored as alternatives to animal models, including the assessment of chemical–protein reactivity. Like skin allergens, respiratory chemical allergens or their metabolites have electrophilic properties and need to react with nucleophilic amino acids in skin- or lung-derived proteins for the effective induction of sensitization. An *in chemico* quantitative peptide reactivity assay based on lysine and cysteine, which are most often cited, is under development for screening chemical skin sensitizers, and this assay may also be used for testing respiratory chemical sensitizers to assess whether their reactivity with peptides is different from chemical skin allergens [14, 18–23]. One important consideration in this approach is that many chemicals exist as prohaptens, which require metabolic conversion to become protein reactive. Therefore, such assays should consider incorporation of a metabolic activation step [24].

In Vitro Cell-Based Models to Assess Sensitization

As skin and respiratory sensitizations are complex immunological disorders, many cell types are involved in their pathogenesis and each undergoes significant changes during these processes. In developing a screening assay for assessment of a chemical's sensitization potential based on *in vitro* technology, the choice of appropriate cell types and mechanism-based endpoints is therefore important. Different experimental approaches have been used to understand the underlying mechanisms and to

assess the potential hazardous effects of LMW industrial compounds. While such *in vitro* methods fully represent an implementation of the vision on toxicity testing in the twenty-first century, there are also challenges to be identified and obstacles to be overcome. The reliance on *in vitro* methods present the long-debated problems with this approach, including the role of metabolism, the ability to extrapolate *in vitro* concentrations to relevant *in vivo* doses, the ability to understand organ interactions, the ability to detect epigenetic changes and other unknown mechanisms, and the fact that cell lines may have some abnormal biology [25].

Various cell types are involved in skin allergy and respiratory allergy, among which epithelial cells, dendritic cells (DC), and macrophages are the most important players in the sensitization phase [26, 27], and are therefore a good starting point for the development of such a test system. An adequate *in vitro* model should be simple, reproducible, quantifiable, relevant for the human situation, amenable to validate, and accepted by industry. Primary cells derived from human skin or lung are relevant cells in this context because they mostly resemble the real-life situation. The disadvantages of such cell systems are donor variability, and they are difficult to obtain in sufficient number and to maintain. In contrast, immortalized cell lines represent valuable models because of their high availability, reproducibility, and standardization potential. A cell line originates from a single donor, and therefore, donor-to-donor variation is not applicable. However, it should be noted that variations can be introduced within a cell line by deviating from the standard protocol or by culturing the cells at high passage number. Because most of them are readily available from various cell and tissue companies, large numbers of cells can be grown in a short time.

Many of the single cell-based *in vitro* assays will only be able to assess one aspect of the multistep process required to achieve sensitization. Recently, progress has been made in combining various cell types into coculture and *ex vivo* models (e.g., precision-cut lung slices) [28–30], which is a good step forward to an *in vivo*-like situation. Also air-lifted, three-dimensional human skin epidermis and lung epithelium models have been reconstructed and are being tested for their capacity to discriminate sensitizing from nonsensitizing chemicals [31–33]. These models are fully in their infancy and additional research is necessary.

Besides the choice of an appropriate cell model, it is also important to study relevant endpoints in the context of skin and respiratory sensitization. New tests should be based on the mode of action of chemicals and should focus on toxicity pathways, as proposed by the NRC. For this purpose, transcriptomics, proteomics, and metabolomics are promising tools that may provide more sensitive, mechanism-based biomarkers. For epithelial cells, DC, and macrophages, gene expression profiles derived in global gene expression studies may shed a light on the pathways that are affected by immunotoxic compounds in general and immunotoxic compounds of different potency in particular. In contrast to classical cellular endpoints which mostly have a limited dynamic range, highly specific biomarkers may be inferred using signature expression profiles that are able to classify chemicals according to their sensitizing potential and thus aid in the hazard identification of existing and novel chemical compounds.

Another question to be solved is the possibility to distinguish between contact and respiratory sensitizers. Given the general similarity of the sensitization response in skin and respiratory allergy, it has been suggested that the advances made in the safety assessment of skin sensitization might be used in concert with, or as the basis for, novel approaches for the assessment of respiratory sensitization potential. However, due to the known mechanistic differences and the more serious health and regulatory implications for classification as a respiratory allergen, the accurate identification of these compounds and their distinction from compounds inducing contact allergy is critical. The use of “omics” tools might help in this context.

A state of the art of the different *in vitro* human cell-based approaches to identify contact and respiratory sensitizing capacity, which among other alternative methods (*in silico*, *in chemico*) are proposed as part of an integrated testing system [34], is described below. For a comprehensive overview, the reader is referred to [8, 9, 31, 35, 36].

Skin Sensitization

Allergic contact dermatitis (ACD) is the manifestation of an allergic response in susceptible individuals caused by skin contact with an allergen and is the most prevalent form of immunotoxicity found in humans. Clinical symptoms of ACD include red rash, blisters or wheals, and/or itchy, burning skin usually confined to the area of allergen contact. The origin and nature of compounds able to induce ACD is very diverse, but all share some common features that govern skin penetration and uptake by immunogenic cells. The first barrier a chemical must cross in order to behave as a chemical skin sensitizer is the *stratum corneum*. To facilitate penetration through the viable layers of the outermost part of the skin, the epidermis, chemicals, generally denoted as haptens, should be hydrophobic (Log $K_{o/w}$ values in the ranges of -3 to 5) and small (molecular weights less than 500 Da) [37, 38]. Since these molecules are too small to be antigenic themselves, haptens readily conjugate to epidermal and dermal molecules. These then act as carrier proteins to form the hapten–carrier complex that finally acts as the antigen. Most haptens bear electrophilic residues which account for covalent bonds to the nucleophilic residues of cutaneous proteins [26, 39]. Of note is that not all sensitizers generate covalent interaction, e.g., metal ions interact via protein–metal chelate complexes, and recent evidence suggests that noncovalent interactions with the major histocompatibility complex (MHC) constitute a novel type of hapten epitope [40]. Haptens often derive from unstable chemicals, the so-called prohaptens, which need an additional metabolic activation step *in vivo* in the epidermis to be converted into an electrophilic hapten endowed with antigenic properties [41].

ACD is a form of delayed-type hypersensitivity (DTH) reaction and is dependent upon cell-mediated immune function and the activity of T lymphocytes [26, 42, 43].

The pathophysiology of ACD consists of two distinct phases: the sensitization phase also referred to as the induction phase and the elicitation phase.

Following first encounter with the chemical sensitizer, during the so-called *induction phase*, a pattern of changes is provoked in the skin to ensure that the immune system is sensitized for an allergic response. Epidermal keratinocytes (KC) constitute an important player in the induction and maintenance of inflammatory cells within the skin through the production of a variety of immunological active cytokines/factors and expression of HLA molecules and cell adhesion molecules. Cutaneous dendritic cells, represented by Langerhans cells (LC) forming a contiguous network within the epidermis [44] and the dermis [45], constitute the true initiators and modulators of immune responses [46]. They form a system of antigen-presenting cells (APC) which exhibit all accessory cell functions, i.e., high expression of MHC (major histocompatibility complex) molecules mediating antigen presentation, as well as of cellular adhesion and costimulatory molecules [47, 48]. LC reside as relatively immature DC characterized by a high capacity to internalize and process skin-encountered antigen. Following antigen uptake, APC become activated and migrate via the lymphatic system to the regional lymph nodes (LN) where they present antigens to T cells [49]. Upon activation, LC upregulate the expression of specific surface molecules (e.g., MHC), adhesion molecules (e.g., CD54), costimulatory molecules (e.g., CD80, CD86), cytokines, and changes in chemokine receptor levels (e.g., CCR1, CCR4) [50–52]. These alterations of the cell's appearance are referred to as the DC maturation process. Following skin contact with the antigen, well-characterized molecular events regulate the interplay between KC and LC. IL-1 β [53], TNF- α , and GM-CSF [54, 55] facilitate migration of LC toward the lymph nodes [56]. Along with their migration and settling within the draining lymph nodes, they adopt a strongly veiled, interdigitating appearance, thus maximizing the chances of productive encounters with naïve T lymphocytes. To acquire clonal expansion of T cells, three distinct signals induce their activation. These include triggering of T-cell receptor molecules (TCR) by MHC molecules, costimulation directed by increased expression of costimulatory molecules on the surface of LC, and stimulation by several cytokines [40]. Upon activation, allergen-specific T cells start secreting IL-2, a potent T-cell growth-promoting factor, and thereby inducing abundant T-cell proliferation [57]. The progeny of primed T cells subsequently sets free via efferent lymphatic vessels and the thoracic duct into the blood [58]. Memory T cells can again leave the circulation and go into lymphoid organs anywhere in the body, thus rapidly ensuring systemic memory.

Once sensitized, individuals can develop ACD with manifestation of clinical symptoms (erythema and induration with a climax within 2–3 days) upon reexposure to the contact allergen during the so-called elicitation phase. After renewed contact, the antigen is taken up by APC as in the sensitization phase which then quickly migrate into regional lymphoid tissue and en route present antigen to sensitized memory T cells within 4–8 h. Cytokines such as IL-1 β and TNF- β , as well as chemokines, are released by activated keratinocytes, leading to the recruitment of antigen-specific memory T cells from the blood. Subsequently, infiltrating cells consisting of monocytes, DC, and both CD4⁺ and CD8⁺ T cells enter the

epidermis and dermis and further amplify the inflammatory response by the production of chemokines and cytokines [45, 59].

In Vitro Assays to Assess Skin Sensitization Hazard

The development of a screening assay based on *in vitro* technology is perhaps the most intensively investigated field in skin sensitization toxicology. Below, different cell types studied for their capacity to distinguish skin sensitizers from nonsensitizing substances are outlined together with the various endpoints that are looked at.

At the Epithelial Barrier

KC are the predominant cell type in the skin and the first cells encountered by chemical exposure. Although their potential to produce discriminating cytokine profiles and oxidative stress responses in KC models, such as the human keratinocyte cell line HaCaT, upon skin sensitizing exposure has been described, the number of studies to develop predictive assays is still confined [60–63]. In general, it seems that studies with keratinocytes prove to be helpful in understanding the immune response to sensitizers and irritants but that the response is rather nonspecific. It looks like keratinocytes signal “danger” and in this way activate different elements of the immune system [64, 65]. Yet, recently, it has been established that primary KC and HaCaT may add significant value to an *in vitro* model for screening skin sensitizers as they allow assessment of the qualitative contribution of epidermal phase I/phase II enzymes involved in xenobiotic metabolism [66].

The Dendritic Cell

Due to their central role during the induction process, DC were rapidly considered as an obvious opportunity for developing *in vitro* approaches for the detection of potential skin sensitizers [67]. Obtaining a sufficient number of human DC from skin to be routinely used in screening assays for skin sensitization remains extremely difficult. Advances in the *in vitro* generation of dendritic-like cells from human sources have led to valuable alternatives, mimicking *in vivo* skin DC. Two main primary-derived DC-like cell models exist, generated either from human peripheral CD14⁺ blood monocytes (CD14-DC) or from cord blood or bone marrow-derived CD34⁺ hematopoietic precursors (CD34-DC) [68, 69]. In order to circumvent the limitation of donor variability, researchers have explored the use of DC-like cell lines as *in vitro* predictive models for skin sensitization [70]. A number of human dendritic-like cell lines have been investigated, including the human myeloid cell lines KG-1 [71], THP-1 [72, 73], U937 [74], and MUTZ-3 [75].

One of the earliest and most extensively studied endpoints to identify sensitizers has been the altered expression of cell surface markers on DC during their maturation. Currently, well-known DC cell surface maturation markers are being scrutinized, and significant expression changes of HLA-DR, CD80, CD83, CD86, CD54, and CCR7 have been reported frequently in both primary DC and DC-like cell lines [67, 70, 76–89]. Of all these possible biomarkers, increased CD86 expression has been examined most extensively and appears to be most promising. The human Cell Line Activation Test (h-CLAT) is a promising alternative method and examines the level of CD86 and CD54 expression on the surface of THP-1 cells, a human monocyte leukemia cell line [90]. The h-CLAT has been evaluated by five independent laboratories in several ring trials coordinated by the European Cosmetics Association (COLIPA). The overall accuracy of 84% indicated a good prediction performance of the h-CLAT [91].

Upon DC maturation, changes in cytokine secretion also occur. To this end, multiple studies have been conducted in which the production of cytokines was explored as possible endpoint in an *in vitro* assay to identify chemical skin sensitizers. The most extensively studied cytokines and chemokines in DC-based assays are IL-1 β , IL-12, IL-18, TNF- α , CXCL8, and CXCL12 [67, 78, 80, 81, 84, 85, 89, 92–101]. Despite the numerous attempts, studies based on the expression of surface markers and cytokines have indicated that their response patterns may be chemical dependent and therefore do not allow the identification of all sensitizers and that they have a limited dynamic range [102].

As a more recent approach, transcript profiling has been applied for detecting genomic fingerprints that are induced in both monocyte-derived dendritic cells (Mo-DC) and CD34-DC models by a single or a group of chemical sensitizers, leading to potential novel and unique biomarkers of interaction of primary DC with sensitizers [103–108]. High-density microarray analyses performed in Mo-DC after treatment with the sensitizer dinitrobenzene sulfonic acid allowed to derive a list of potential target genes [107], whereas similar analyses performed on CD34-DC exposed to our known sensitizers and two irritants revealed 119 significantly discriminating genes [109]. In both Mo-DC and CD34-DC biomarkers identified by transcriptomics, analyses were further confirmed in an expanded chemical test set by performing real-time RT-PCR [110, 111]. In the CD34-DC model, these experiments have led to the development of a prediction model (VITASENS®) for skin sensitization potential based on the differential response of C-C chemokine receptor 2 (*CCR2*) and cAMP-responsive element modulator (*CREM*) to an expanded set of 21 (non)-sensitizing chemicals (Fig. 1). So far, this primary DC-based alternative test showed a high predictive power with a concordance of 89%, specificity of 97%, and sensitivity of 82% at the level of donor samples [111]. A recent study indicated that in the THP-1 cell line, the expression pattern of biomarkers identified in primary CD34-DC appeared to be less discriminating toward (non)-sensitizing exposure [112].

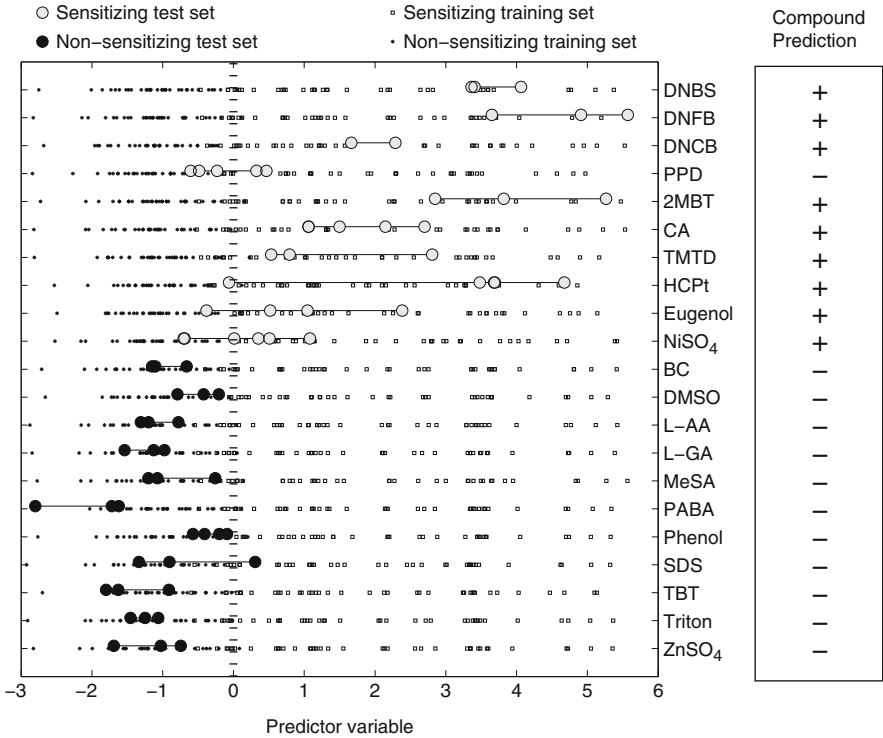


Fig. 1 Comparison predictions of the VITOSENS® model in a cross-validation. Per test compound, each circle corresponds to a donor sample and is represented by its predictor value: positive = sensitized, negative = nonsensitized. The right panel gives the prediction per compound by applying a majority rule on the representing test samples: + = sensitizer, - = non-sensitizer. Abbreviations: *DNBS* dinitrobenzenesulfonic acid, *DNFB* dinitrofluorobenzene, *DNCB* dinitrochlorobenzene, *PPD* *p*-phenylenediamine, *2-MBT* 2-mercaptobenzothiazole, *CA* cinnamaldehyde, *TMTD* tetramethylthiuram disulfide, *HCpt* ammonium hexachloroplatinate IV, *Eug* eugenol, *NiSO₄* nickel sulfate, *BC* benzalkonium chloride, *DMSO* dimethylsulfoxide, *L-LAA* L-ascorbic acid, *L-GA* L-glutamic acid, *MeSA* methyl salicylate, *PABA* *p*-aminobenzoic acid, *SDS* sodium lauryl/dodecyl sulfate, *TBT* tributyltin chloride, *Triton* Triton X-100, *ZnSO₄* zinc sulfate

T-Cell Assays

The activation and clonal expansion of allergen-responsive T cells represents the central event in the acquisition of skin sensitization. A relevant target for *in vitro* screening of skin sensitizers would therefore be detecting the specific response among naïve T lymphocytes as provoked by skin-sensitizing chemicals. One research group has reported discrimination of a first selection of sensitizers from nonsensitizers based on the induction of T-cell proliferation after exposure of cocultures of CD14-DC and autologous T cells [113]. To date however, no progress was reported from such assays. The challenge in this method lies in the identification

of what is likely to be a weak signal against a rather noisy background and the interference of considerable donor variation. Recently, a novel optimization protocol for the induction of T cells has been published which is free of exogenous growth factors [114]. This assay which is developed in the context of vaccine evaluation could be an opportunity for developing the first *in vitro* assay to identify skin sensitizers based on T-cell stimulation.

Coculture and 3D Models

Coculture systems and more sophisticated models created in order to reconstruct the typical skin layers found *in vivo* are gaining interest in the development of screening assays for skin-sensitizing chemicals.

Besides the model integrating LCs derived from CD34⁺ progenitor cells into reconstructed human epidermis [115, 116], another example that was published recently is the “loose-fit coculture-based sensitization assay” (LCSA). This model is composed of a single layer of primary human KC and of allogenic free-floating monocytes, which differentiate to DC-like cells in the presence of exogenous cytokines [117, 118]. Based on the stimulation of CD86 expression and cell viability of DC after exposure, the skin-sensitizing potential of chemicals could be estimated [30]. Such immunocompetent tools are currently being developed in a larger European Commission-sponsored integrated Framework 6 project (Sens-it-iv: <http://www.sens-it-iv.eu>).

Today, several commercial providers propose reconstructed skin or epidermis, which have been validated as *in vitro* assays for skin irritation, e.g., the EpiDerm™ skin model (MatTek, US) and the EPISKIN™ reconstituted human epidermis model (RHE) (SkinEthic, L'Oréal, France) [119]. *In vitro* assays for chemical sensitization based on these 3D models are still under development [120]. Levels of extracellular IL-1 α and IL-8 in the RHE have been studied for the prediction of skin irritation and/or sensitization [121].

Mechanistic Role of Skin Sensitization Biomarkers

Although a lot of promising *in vitro* assays are being developed, it should always be kept in mind that since the desire is to have a test system that incorporates an understanding of the biological mechanisms of the induction of skin sensitization, it will be necessary and appropriate to consider the biological relevance of selected candidate genes [122]. Linking the identified biomarkers as functional players in the complex process of skin sensitization will emphasize the relevance of the *in vitro* assay for screening possible human skin-sensitizing chemicals.

When an *in vitro* assay incorporates the complex, underlying molecular events of skin sensitization, it will aid in a better extrapolation of data and an improvement of risk assessment. Therefore, linking assay markers as functional players in the

complex process of skin sensitization will emphasize the relevance of the *in vitro* test for screening possible human skin-sensitizing chemicals.

One such an example is the biological characterization of marker genes in the VITASENS® assay [123]. Using pathway analysis software, a literature-based network was constructed around VITASENS® markers, in which the presence of other skin sensitization-related molecules was revealed. Effective transcription of a small selection of these network members in CD34-DC was shown by their differential expression after sensitizing chemical exposure. In a first attempt to prove the functional relevance of VITASENS® marker genes, the significantly differential protein expression of CCR2 and COX2 was established in sensitizer *versus* non-sensitizer-exposed CD34-DC. By proving the protein expression pattern of the marker genes to be equally discriminating compared to the gene expression responses, the relevance of the VITASENS® discriminatory reaction for the biological process by which DC activate and present chemical haptens to naïve T cells is illustrated.

The KeratinoSens assay is a recently developed pathway-based assay that also fits the paradigm of toxicity testing in the twenty-first century. A reporter construct with luciferase activity was incorporated in a KC cell line, and upon exposure to skin sensitizers, the NF-E2-related factor 2 (Nrf2) transcription factor is activated and binds to antioxidant response element (ARE) in the promoter sequence of cytoprotective genes, thereby emitting quantifiable light [61]. The development of a pathway-based assay in a high-throughput setup may significantly enhance the rapid and efficient screening of chemicals for their possible skin-sensitizing properties.

Potency Assessment In Vitro

Improving the disease mechanism aids not only in the development of more precise prediction assays but also in a better extrapolation of the data for more accurate calculations of the risk for humans. As is the case for many diseases, skin sensitization is not an all-or-none phenomenon. There is evidence that dose–response relationships exist [124]. Appropriate dose–response data can provide important information on the potency of the material being tested, which may facilitate the development of more accurate risk assessments. Such data can now be generated from the mouse local lymph node assay (LLNA) for the sensitization phase, but it must be remembered that these animal data cannot be translated directly to human thresholds [125–127].

Earlier reports suggested that genes expressed in primary DC might be capable of classifying allergens into specific potency categories based on relative differences in gene expression [111]. Similar findings could be observed in the VITASENS® assay. In this primary CD34-DC model, the gene expression dataset was extended by another *in vitro*-generated variable, namely, the IC₂₀ or the concentration of the test chemical that yielded 20% cell damage. The relevance of including this IC₂₀ as an explanatory variable was experimentally demonstrated by a clear

viability of DC after exposure as a measure of skin-sensitizing potential of chemicals [30]. Also, in the DC-like cell line, THP-1 attempts to correlate *in vivo* potency data and *in vitro* predictions have been published [90, 131].

These findings may have major implications on human risk assessment. Potency information may contribute in the derivation of no- or minimal-effect levels, which is the threshold required for the induction of skin sensitization. Identification of maximum usage levels would be a clear step forward toward achieving increased chemical hygiene.

Lung Sensitization

A respiratory sensitizer is a substance that can induce a type 1 hypersensitivity reaction of the airways following inhalation of the substance. Hypersensitivity reactions induced by respiratory sensitizers may result in asthma, which is clinically characterized by wheezing, breathlessness, tightness in the chest, bronchoconstriction, and/or nasal congestion as most common symptoms. Asthma attributable to stimuli encountered at the workplace is defined as occupational asthma (OA), which is considered the most common occupational lung disease.

More than 350 agents have been implicated in the onset and development of respiratory allergy. A complete list of those agents can be found in research articles and reviews [132–138]. These agents are categorized into high molecular weight (HMW) and low molecular weight (LMW) agents.

The HMW compounds are generally of biological origin, e.g., proteins derived from animals, plants, foods, and enzymes, and act as complete allergens. Exposure to HMW agents is recognized by activation of antigen-presenting cells, leading to the production of specific IgE antibodies by B cells. Cross-linking of allergens with a specific IgE antibody on the surface of mast cells, basophils, and possibly macrophages, dendritic cells (DC), eosinophils, and platelets gives rise to a cascade of events that result in the influx and activation of inflammatory cells and in the release of preformed and newly formed inflammatory mediators that orchestrate the inflammatory process [27, 139, 140].

LMW compounds are generally chemically synthesized and represent an important subset of etiologic agents, including approximately 100 separate chemical entities. Although there are fewer LMW chemicals than HMW agents in the lists of occupational respiratory sensitizers, recent data indicate that LMW chemicals account for more new cases of OA caused by sensitization [17]. LMW chemical agents probably act as haptens becoming allergenic after conjugation with one or more body proteins [141]. Some of these induce asthma through a typical allergic Th2-type mechanism with attendant IgE production. The most well-known compounds in this group include acid anhydrides, metals, reactive dyes, and some pharmaceutical products. The majority of LMW compounds, however, do not consistently induce specific IgE antibodies, and the pathogenesis remains only partially elucidated [139, 141–144]. Specific IgE and IgG antibodies are found in

only a minority of cases and their precise role is unclear [145]. The airway inflammation process, however, is similar in IgE-dependent and IgE-independent asthma [146, 147] and is characterized by the presence of eosinophils, lymphocytes, mast cells, and thickening of the reticular basement membrane. Asthma due to diisocyanates, western red cedar, and amines are examples of IgE-independent causes of OA [148].

In Vitro Assays to Assess Respiratory Sensitization Hazard

As mentioned above, epithelial cells, DC, and macrophages are key cells involved in the development of respiratory sensitization.

The Epithelial Barrier

Bronchial and alveolar epithelial cell models have been extensively used to study the inflammatory potential of xenobiotics [149–161]. These models are good candidate assays to assess their use for respiratory sensitization screening. Recently, attempts to develop a transcriptomics-based *in vitro* assay based on alterations in gene expression of human epithelial cells after exposure to respiratory sensitizers and respiratory nonsensitizing chemicals have been initiated in order to identify genes that are able to discriminate between both groups of chemicals.

In the human bronchial epithelial BEAS-2B cell line, several genes have been identified that can distinguish three respiratory sensitizers from two irritants and one skin sensitizer [162]. Among the top-20 selective marker genes, *CASP9* is an interesting gene that can be associated with asthma and/or respiratory sensitization. When categorizing the 1,000 most discriminative genes into biological GO terms, 20 genes were associated with immune function. The majority of these genes have been shown to be related to airway inflammation, asthma, and respiratory sensitization (e.g., *CCL24*, *CEBPB*, *MIF*, and *TLR4*). Within this cellular model, the phosphatase and tensin homolog (PTEN) signaling pathway was identified as specific for respiratory sensitization.

In a similar study using the A549 alveolar epithelial cell model, a ranking of genes was obtained that reflected their potential to discriminate between respiratory (non-)sensitizing chemicals (e.g., *CLGN*, *CTLA4*, and *TXLNA*). Of these, *CTLA4* was associated with immune responses. When categorizing the top-1,000 genes into biological Gene Ontology (GO) terms, 22 genes were associated with immune function (e.g., *CCL4*, *CCL26*, *SPPI1*, and *TLR2*). In this study, none of the canonical signaling pathways activated in alveolar epithelial cells were specific for respiratory sensitization [163].

The Dendritic Cell

Although airway DC are believed to be the conductor of immunological response, there is a growing awareness that these cells are activated by the presence of local immunomodulatory signals. A substantial proportion of these signals originate in the airway epithelium. DC also interact with epithelial cells by expressing adhesion molecules that not only anchor them strongly in the epithelium but also have a possible effect on their function [129, 130, 164–166].

Several DC-based assays (CD34-DC, Mo-DC, MUTZ-3, THP-1, KG-1, and U937), which have initially been developed for the purposes of skin sensitization testing [70], have been further exploited for respiratory allergen identification in the European Sens-it-iv program [97, 167, 168].

Macrophages

In the lung, resident alveolar macrophages are continuously encountering inhaled substances due to their exposed position in the alveolar lumen. Macrophages play a central role in the response to foreign materials and exhibit effects on alveolar and interstitial DC and T cells [169]. Besides *ex vivo* isolated alveolar macrophages, different human cell lines (THP-1, Mono-Mac-6) have been studied as a model of lung-derived macrophages [170–172].

Using THP-1-derived macrophages as *in vitro* model, different marker genes have been identified using gene expression studies that can distinguish respiratory sensitizers from respiratory nonsensitizers (e.g., *EIF4E*, *PDGFRB*, *SEMA7A*, and *ZFP36L2*). The function of the 1,000 most discriminative genes was linked to diverse biological processes, and 24 genes were associated with immune responses (e.g., *CCL20*, *IL10*, *TNF*, and *TLR4*). It could also be concluded that the canonical platelet-derived growth factor (PDGF) signaling pathway is probably the most specific one that was observed to be activated in macrophages in the context of respiratory sensitization, which is supported by existing literature information [173].

Coculture and 3D Models

A new area of research is the development of coculture models, and some of these have successfully been used for investigating both direct and indirect interactions between xenobiotics (e.g., cigarette smoke, ozone, and particles) and different cell types to mimic naturally occurring exposures. In the future, such culture systems may provide markers of immunoprocessing interactions.

Human monocytes and THP-1 macrophages were cocultured with monolayers of epithelial cells (human BEC, A549, renal adenocarcinoma epithelial cells) and the lung fibroblast strain HFL-1 [174]. A triple coculture model including macrophages and epithelial and dendritic cells which mimic the physiological communication

between these cell types has been developed [32] and might give new opportunities for sensitization screening of chemicals. Other examples of cocultures are BEAS-2B with human lung fibroblasts (HFL-1 or WISTAR-38) [175, 176], human umbilical vein endothelial cells (ECV304) [177], or eosinophils [153], and primary human BEC with alveolar macrophages [178].

In addition, 3D models of human airway tissues have been generated in order to study respiratory toxicity and infection. When cultivated *in vitro* on a polycarbonate filter at the air–liquid interface in a chemically defined medium, the human A549 alveolar cell resembles histologically the outer alveolar cell layers of the human lung alveoli (SkinEthic Laboratories, Nice, France). This model can be used for efficacy profiling of compounds or drugs for asthma treatment or type 1 allergy, or to better understand the safety potential of developed products applied by inhalation. Furthermore, Aufderheide *et al.* (2003) [179] reported on a technology allowing exposure of cells in a 3D culture to well-characterized aerosols (CUL-TEX®). MucilAir™, a novel *in vitro* cell model of the human airway epithelium, is a tool for acute and long-term toxicity testing of chemicals (Epithelix, Geneva, Switzerland). This *in vitro* system closely mimics the morphology and functions of the native human airway epithelium. There is little variation between batches that guarantees the reproducibility of the tests, and it has the ability to regenerate and repair after mechanical and chemical injuries.

Prospects

Chemical-induced allergy remains an important occupational health issue and a challenge to toxicologists. Although a considerable investment has been made in exploring opportunities to develop methods for hazard identification and characterization, there are, as yet, no validated alternative (*in silico*, *in chemico*, *in vitro*) methods available. Nevertheless, a number of emerging *in vitro* and *in silico* models show promise for use in the characterization of contact sensitization potential and should be further explored for their ability to identify and differentiate contact and respiratory sensitizers. Some of these systems may be useful in a weight-of-evidence approach or as a preliminary screen.

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