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Perspectives on Non-Animal Alternatives for Assessing Sensitization Potential in Allergic Contact Dermatitis

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Abstract

Skin sensitization remains a major environmental and occupational health hazard. Animal models have been used as the gold standard method of choice for estimating chemical sensitization potential. However, a growing international drive and consensus for minimizing animal usage have prompted the development of *in vitro* methods to assess chemical sensitivity. In this paper, we examine existing approaches including *in silico* models, cell and tissue based assays for distinguishing between sensitizers and irritants. The *in silico* approaches that have been discussed include Quantitative Structure Activity Relationships (QSAR) and QSAR based expert models that correlate chemical molecular structure with biological activity and mechanism based read-across models that incorporate compound electrophilicity. The cell and tissue based assays rely on an assortment of mono and co-culture cell systems in conjunction with 3D skin models. Given the complexity of allergen induced immune responses, and the limited ability of existing systems to capture the entire gamut of cellular and molecular events associated with these responses, we also introduce a microfabricated platform that can capture all the key steps involved in allergic contact sensitivity. Finally, we describe the development of an integrated testing strategy comprised of two or three tier systems for evaluating sensitization potential of chemicals.

Keywords

Immunobiology; Skin sensitization; *In silico* approaches; QSAR; 2D cell based models; 3D skin tissue models; Integrated Testing Strategies; Microfabrication; Microfluidics

INTRODUCTION

Approximately 15–20% of the population in the Western world and ~1% of the worldwide population is allergic to one or more environmental chemicals.[70] According to the National Center for Health Statistics (NCHS), in the United States alone, approximately 9% of the

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population in the 0–18 year age range suffers from allergic contact dermatitis, (ACD) with even higher risks in the adult population. While ACD is not a medical emergency, the risks associated with these conditions can be very distressing, causing great discomfort, emotional stress and feelings of hopelessness, and carry a huge economic burden due to the costs of medical care and lost productivity.[34]

ACD is a delayed hypersensitivity reaction (Type IV) mediated by antigen-specific T lymphocytes in sensitized patients exposed to contact allergens such as nickel, nickel sulfate, 2,4-dinitrochlorobenzene, paraphenylenediamine, cinnamic alcohol and formaldehyde. [87] Typically, a chemical is classified as a sensitizer allergen if it induces allergic contact sensitivity, or an irritant if it does not elicit a cytokine secretion profile that is responsible for T cell proliferation within the draining lymph node, but evokes non-specific skin inflammation.[4] Metals such as nickel and gold used in medical implants are the most commonly known sensitizers [148] that elicit an inflammation mediated cytokine release that is pivotal for allergen specific T cell responses. Nickel sulfate has also been shown to induce secretion of cytokines such as IL12p40 that play a role in activating allergen or sensitizer specific T-cell response.[6] 2,4-dinitrofluorobenzene and nickel sulfate have been shown to upregulate surface markers such as CD40 and IL-12 receptor that are implicated in the sensitizer mediated T cell response in fetal dendritic cell lines[163] while 2,4-dichloronitrobenzene, an irritant shows no effect. Previous research work has also been focused on generation of nickel specific T-cell lines that evoked a proliferative response specific to haptens.[105]

Paraphenylenediamine, a permanent hair-dye product highly susceptible to oxidation, has been shown to selectively bind to cysteine residues in skin peptides and proteins prior to eliciting a T-cell response.[67] Moreover, the chemical, either alone or in combination with an oxidant induces B cell and T cell infiltration within the draining lymph nodes in mice.[21] Cinnamic alcohol is a weak sensitizer, but, upon contact with skin, an epidermal enzyme mediated metabolic conversion to cinnamaldehyde elicits an allergen response.[11]

The overall immune response to allergens is comprised of a complex cascade of events as shown in Fig. 1. Topical application leads to diffusive penetration and distribution of allergens within skin wherein it reacts with extracellular or cell membrane bound proteins to form immunogenic complexes capable of inducing T cell responses.[38] Exposure of allergen leads to maturation and migration of skin-resident Langerhans cells (LCs) and dermal dendritic cells (DCs) to the localized lymph node. The activation and migration of DCs is regulated by multiple factors including chemokines and cytokines secreted by various resident skin cells.[75,76] The maturation results in the LC/DCs acquiring antigen, expressing co-stimulatory molecules, and expressing chemokine receptors such as CCR7.[141] The migration of LC/DC is believed to be regulated by chemokine gradient of CCL19 and/or CCL21 across the lymphatic vessel.[129] DCs play a central role in transporting the antigen to the localized lymph node, where they initially form immunological synapses with T cells, ultimately leading to T cell activation. The activated T cells undergo differentiation and proliferation, which results in the selective expansion of clonal populations of allergen-specific T cells. The activation of Th1 and/or Th2 clonal T cell populations ultimately results in allergen-specific sensitivity.

In order to both predict skin allergy responses and unravel the complexity of these responses, the murine local lymph node assay (LLNA) was developed using US FDA and the Organization for Economic Cooperation and Development (OECD) guidelines. Although *in vivo* assays like the murine LLNA have been extensively used to predict sensitization potential, both the pressing social need to replace animal sensitization models, and the scientific need to more precisely control and evaluate the mechanistic progression of hypersensitivity have spawned interest in the development of *in vitro* models. Moreover, animal models are expensive and require large animal numbers for rigorous chemical testing. In the US, there are rough estimations that about 10 million animals per year are used in toxicity testing of chemicals and drugs. In the EU, as of 2005, approximately 1 million animals were utilized for end-point toxicological studies.[94] In the frame of the new EU-Chemicals directive Registration, Evaluation, Authorization of Chemicals (REACh),[119] there are estimates that about 40–50 million additional animals may be required in order to fulfill all related obligations in toxicological characterization and risk assessment of the so called 30,000 "existing chemicals" that have entered the market before 1981.[48,53] Moreover, the recent 7th Amendment to the European Union Cosmetics Directive (2003/15/EC) is set to ban all *in vivo* testing of toiletry and cosmetic ingredients and the marketing of such products within the European Union (EU) by 2013. Furthermore, this ban is likely to be effected in the US in subsequent years, as animal tested products may be at a distinct marketing disadvantage. Moreover, while the LLNA is a robust method to capture the complexity of skin allergies, the translation of animal model data to human systems is not entirely predictive. All of these factors necessitate the development of non-animal alternatives for chemical testing.

Current non-animal alternatives for predicting sensitization potential include *in silico* approaches involving the incorporation of experimental information into sophisticated computational modules to generate Quantitative Structure Activity Relationships (QSAR) i.e. skin sensitizer activity as a function of the chemical structure. These QSAR based models include expert systems such as the Deductive Estimation of Risk from Existing Knowledge (DEREK), Toxicity Prediction by Komputer Assisted Technology (TOPKAT) and Times Metabolism Simulator platform used for predicting Skin Sensitization (TIMESSS) systems.[71] Other approaches include a mechanism based read-across model (that takes into account compound electrophilicity for sensitization potential assessment) and assessment of simple sensitization endpoints based on peptide-binding potential of chemicals. However, these assays do not recapitulate the cellular elements of the more complex *in vivo* events that take place in the skin and the draining lymph nodes such as maturation and migration of DCs from the skin to the lymph node where they present antigens to naive T cells. In order to address some of these issues, cell and tissue based assays have been developed that include a variety of mono and co-culture cell systems in conjunction with 3D skin models. Given the complexity of allergen triggered immune reactions, and the limited ability of existing systems to capture the entire gamut of cellular and molecular events associated with these immune responses, we also describe a microfabricated platform that can capture all the key steps involved in allergic contact sensitivity. Finally, we describe the development of an integrated testing strategy comprised of two or three tier systems for evaluating sensitization potential of chemicals.

In the paragraphs below, we discuss in detail, the approaches mentioned above that have been developed to recapitulate the sensitization process (Figs. 1 and 2) and future implications for an integrated microfluidic system to clearly mimic the *in vivo* ACD phenomena using non-animal alternatives.

IN SILICO APPROACHES

Many *in silico* approaches exist that predict the degree of skin sensitization based upon the chemical structure of chemical entities. For example QSAR as well as its derivatives, such as DEREK and TOPKAT systems, and alternatives such as mechanism based read-across models, have had similar impacts in predicting potential skin sensitization. In the following sections we will briefly describe these approaches.

(Quantitative) Structure Activity Relationships (QSAR)

Hansch and Fujita were the first to introduce the concept of Quantitative Structure Activity Relationship (QSAR). The research highlighted the relationship between the molecular structure and biological activity of a molecule. The basic assumption for all molecule based hypotheses was that similar molecules have similar activities. This principle is also called Structure-Activity Relationship (SAR). QSAR was primarily developed to estimate the boiling points of chemicals based on structure of alkenes. This methodology was further advanced for utilization in fields such as small molecule drug discovery,[40] lead optimization[147] and bioresponse data from combinatorial libraries of biomaterials. [145] This approach was derived in the pharmaceutical industry as a means of predicting the solubility, bioavailability, clearance, and cellular uptake of a compound relative to its dose dependant final efficacy and toxicity.[19,23,27,28,71,106,112,122,151,162,164,170] Moreover, QSAR was utilized in combination with Principal Component Analysis (PCA) to predict the mechanism of action of anti-cancer drugs.[85] QSAR has wide applicability in neuroscience for predicting the ability of chemicals to penetrate the Blood Brain Barrier (BBB) based on molecular size and lipophilicity.[24]

For predicting skin sensitization potency of chemicals, various QSAR models have been developed based on heterogeneous diverse chemical data-sets based on experimental conclusions from Local Lymph Node Assay (LLNA), Bundesinstitut für Gesundheitlichen Verbraucherschutz Und Veterinärmedizin (BgVV) or Guinea Pig Maximization Test (GPMT).

The induction phase of skin sensitization, specifically T-cell proliferation within the draining lymph nodes is the central event captured in the most widely utilized and accepted LLNA. In this assay groups of mice are treated with various concentrations of the test chemical applied topically to each ear, and the stimulation of proliferative responses in the draining lymph nodes is measured and compared with the response in control animals. The primary murine method provides a quantitative estimate of the concentration of chemical required to induce a stimulation index of three quantified as the extent of thymidine incorporation in lymph nodes from dosed animals relative to concurrent vehicle-treated controls using linear interpolation. This index is referred to as the EC3 value.[13–17,54]

The BgVV list of 264 chemicals was compiled and evaluated by a group of experts including dermatologists from universities and representatives of the chemical industry and from regulatory authorities that was established by the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) in 1985.[143] Data from the literature on substances with documented contact allergenic properties in humans (from clinical data and experimental studies) and from animal experiments were evaluated resulting in a publication where chemicals were listed as belonging to one of 3 categories (A–C) where category A represented significant contact allergens, B. a solid based indication for contact allergenic potential and C. insignificant questionable contact allergenic potential.

The guinea pig maximization test (GPMT) of Magnusson and Kligman was published in 1969. There are some issues associated with this test. New information is required with regards to the interpretation of challenge results. In particular, overestimation of allergenicity owing to false-positive reactions is common. The control group should be exposed to a chemical insult at induction which provokes an inflammatory reaction comparable to the test substance. Allergic reactions should persist on rechallenge weeks later, while nonspecific irritant reactions generally do not persist and are irreproducible in particular animals. Finally, when a chemical is identified as a contact sensitizer in the GPMT, that result is simply a categorical statement of a theoretical hazard with no risk assessment. In the GPMT, a test substance is defined as a sensitizer if >30% of animals shows a positive response.

The chemical data-sets obtained from *in vivo* tests in animals have been rigorously tested and formulated over the past several years. The LLNA test is unique and comprehensive in providing a quantitative estimate of the sensitization potency of chemicals with relatively fewer animals and less expense as compared to the GPMT. Moreover, this test has been confirmed to have ~70% accuracy as compared to human test data.[59] The positive predictivity of LLNA has been estimated to be >90%. However, the poor performance (<20%) in predicting non-sensitizers reduces the overall accuracy of LLNA. The approach based on chemical data-sets is more prevalent in the recent decades which entails statistical methods such as linear discriminant analysis or linear regression models that link the EC3 (estimated concentration of a chemical required to produce a 3-fold stimulation of draining lymph node cell proliferation compared with concurrent controls) values from LLNA data-sets as biological end-points to structural, topological, electronic or physiochemical descriptors of the molecules.

The Table 2 lists various QSAR models described using linear regression, discriminant analysis, neural networks etc., the number of chemicals tested as sensitizer or non-sensitizer and prominent features of the models. As shown in Table 2, the most comprehensive QSAR model (TIMES-SS) has been developed by incorporating the skin metabolism component into the model by introduction of weight parameters in the model system based on skin enzyme transformation capacity of the sensitizer. TIMES-SS developed at Bourgas University, incorporates skin metabolism and sensitizer structure–activity relationships in a single platform. Using TIMES-SS, there have been significantly consistent relationships between the simulations and certain experimental data for prediction of chemical

sensitization potential. However, the methodology is not completely developed and requires further improvements such as inclusion of diverse reaction chemistry pathways. As indicated, the prediction capability for sensitizer is 65% while for non-sensitizer, it is 72% from a dataset of 634 chemicals (Table 2).[37] The low predictability of the sensitizers is due to 34% prediction of weak sensitizer that results in reduction of the 80% prediction of moderate, strong and extreme sensitizers.

QSAR models based on the guinea pig maximization test predicted skin sensitization for 76.5% of chemicals correctly by taking into account molecular descriptors and 27 structural alerts including the HOMO-LUMO energy gap (that represents the excitability of the molecule based on gap in energy of molecules between the lowest occupied and highest occupied molecular orbits), the Shannon index that accounts for molecular size and structural alerts that relate chemical reactivity sites causing skin sensitization (Table 2).[33] Devillers, 2000 used artificial neural networks to predict accuracy of 83 and 94% for the sensitizers and non-sensitizers, respectively with a non-linear analysis model (Table 2).[35] Extended database of chemicals (up to 300) is also included in Kern *et al.*[73]

More recently, an excellent QSPR model system has been established that takes into account the chemical data-sets from the LLNA, GPMT and BgVV tests.[59] The QSPR model system was developed based on combination of literature and structural descriptors and resulted in 90% accuracy for the LLNA dataset This robust QSPR methodology was also tested against LLNA, GPMT and BgVV databases to obtain a comprehensive output of predictive capacity for each data-set.

TOPKAT as mentioned in Table 2 is multi-stage QSAR model system involved in the identification of sensitizers. The first stage searches for unknown fragments in the chemical to conclude if the molecule is present in the training set. The second stage determines if the structure of chemical is in the prediction space of the model. The third stage develops a probability value of the compound to be a sensitizer or a non-sensitizer. As indicated in Table 2, TOPKAT resulted in 83% prediction of sensitizers for a set of 178 chemicals for the GPMT database.

DEREK for Windows is an expert system that takes into account the toxicity and metabolism of the unknown molecule by comparison with similar molecular structure compounds in the training set. These lead to structural alerts that result in information processing that includes species based endpoint sensitization model (i.e. human, mouse, guinea pig etc.) and physicochemical properties of the molecules. The method provides end-users with a degree of confidence for the chemical to be a sensitizer or not. Multiple computer automated structure evaluation (MULTICASE) is a similar learning platform that further takes into account the mechanistic details of the compound transformations. As indicated in Table 2, DEREK resulted in 73.3% prediction of sensitizers for a set of 178 chemicals for the GPMT database and LLNA database.

For further details on the applicability domain of SAR and QSAR models for skin sensitizers based on chemical sensitizer data-sets using LLNA, GPMT and BgVV, please

refer to literature reviews.[37,55,59,120,160] Various companies have also been developed that provide QSAR based models as listed in Table 2.

While considerable effort has been expended into developing reliable predictive QSAR models, the following considerations may required to be incorporated:

1. Skin metabolic transformation mechanisms are not included in the validation or training datasets of most QSAR models. Incorporation of a step-wise QSAR model domain based on different descriptors could work for predicting sensitization potential of chemicals.[37]
2. The statistical correlation between biological end-points and chemical descriptors is a quantitative formulation that lacks development of multiple hypotheses from a scientific perspective that is critical for accurate prediction of chemicals.[68] For e.g.: the models do not take into account mechanistic details such as electrophilicity and hydrophilicity.[133] As a result, a mechanistic description of the molecular interaction of the sensitizer with a target nucleophilic skin peptide or protein cannot be estimated using these approaches. In a separate section below, we discuss a read-across method that takes into account the action of sensitizers through specific mechanisms.

Mechanism Based Read-across Models

Read-across is a qualitative method based on the concept that the activity of sensitizers, via a Michael addition mechanism, can be utilized to generate an electrophilicity index to categorize sensitization potential using quantitative methods. This approach has numerous advantages over existing QSAR based methods, i.e. ease of understanding and transparency, compliance with OECD regulations for QSAR validation due to a simple mechanistic approach, and ability to make computational interpolations of chemical toxicity in risk assessment platforms.

In a mechanism-based read-across method, the chemical to be tested is screened for structural alerts in accordance with a sensitization mode of action. If a Michael addition mechanism is involved, the global electrophilicity index can be determined as a function of chemical potential and chemical hardness for the specific chemical.

A common metric for distinguishing chemical sensitization potential is the Effect Concentration (EC3) value determined by the classical LLNA. EC3 is the test concentration of a chemical that elicits a 3-fold increase in lymph node T cell number as compared to control sets. Based on plots of the pEC3 (log of the molar EC3 concentration) values for known chemicals vs. the electrophilicity indices, the EC3 value for the unknown chemical can be estimated and compared to existing data using mechanism based read-across.[45] The major limitation of mechanism based read-across is that the methodology requires upper and lower bounds of electrophilicity values of at least two known chemicals for the EC3 of the unknown chemical to be determined. This might not be available in the case of all sensitizers and hence limits the applicability of the method. Also, similar to QSAR models, the mechanism based read-across does not take into account pro-electrophilicity, i.e. the

ability of chemicals to be metabolically activated by skin enzymes prior to becoming electrophiles.

IN VITRO APPROACHES

While *in silico* approaches are promising, a variety of *in vitro* systems also exist to investigate the effect that sensitizers have at the molecular, cellular and tissue levels. In the following sections we will review these approaches.

Protein Binding Assays

Landsteiner and Jacobs[83] developed the first method to correlate simple chemical compounds to their sensitization potential based on formation of covalent adducts with protein molecules.[83] Formation of macromolecular immunogen after chemical reaction of amino acid residues of nucleophilic skin proteins with electrophilic chemical sensitizers is the hallmark of sensitization.[53] Human albumin is the model protein for most of the peptide reactivity assays[53]; approximately 40% of albumin not in vascular tissues is prevalent in the skin.

The peptide reactivity assay is based on the formation of a covalent bond between the sensitizer chemical and keratinocyte peptides or other protein structures in the skin (Fig. 3).[53] This binding event is considered to be a determining step in the initiation of the sensitization process. The assay measures the percent depletion of electron rich Cysteine and Lysine as a function of sensitization potential of the chemicals. The reaction between electrophilic sensitizer chemicals and nucleophilic peptides along with glutathione serves as a platform to predict peptide reactivity of different chemicals.[58] Direct peptide reactivity assay (DPRA) has been tested for 82 chemicals with 89% prediction of sensitizers and non-sensitizers (Table 2).

However, it is estimated that up to 33% of known chemicals as sensitizers are not reactive and require activation prior to interaction with skin peptides to sensitize skin tissue.[56] DPRA does not take into account the skin-derived metabolic component that is clearly imperative for pro-haptens. In this regard, recent work has been focused on inclusion of poorly soluble chemicals and pro-haptens that undergo metabolic activation prior to interaction with skin peptides. The data clearly shows significant increase in peptide depletion (a biological endpoint defined as percent peptide depletion) for pro-haptens using the HRP/HP assay system as compared to routine DPRA.[56] Overall, there is still an urgent need to test a tabulated list of chemicals from various datasets including complex molecular structure and functional groups, skin metabolic transformation capability and unknown reactivity based on standard mechanistic applicability domains. A comprehensive DPRA that tests a wide array of chemicals will provide the final outcome for accurate prediction of sensitizers. Further details of peptide reactivity assays is elucidated in the literature.[80,160]

2D Cell Based Models

A fundamental process in contact allergy is the activation and migration of LCs and dermal DCs followed by T-cell activation. Several investigative groups have targeted primary cells and cell lines in their considerations of appropriate *in vitro* 2D surrogates, whereby they

monitor the increased expression of specific surface markers and signal transduction proteins, cytokine secretion profiles, chemokine induced DC migration, T cell activation and gene expression patterns of DCs amongst others in response to allergen treatment.

In the case of primary cells, human DCs are derived either from CD14+ peripheral monocytes (moDCs)[131] or from CD34+ cord blood cells (CD34+ DCs). Maturation of DCs via sensitizers is measured by expression of IL1*β*mRNA,[43,44] plus a variety of cell surface DC maturation markers such as CD86, CD83, CD207, CCR7 and CD54.[3,4,32,64,155,156] In addition to measuring cell surface marker expression and cytokine production, identifying signal transduction pathways and gene expression patterns that change upon sensitizer exposure is another avenue for assessing sensitization potential.[107,127] Also, the effect of reactive oxygen species on skin sensitization mediated by chemicals has been recently discussed in the literature. [98,101] While primary cells may provide the most suitable cell source, donor to donor variability and their limited availability may preclude their use in high throughput studies employing multiple allergens.

In order to generate DCs for simulating contact hypersensitivity phenomena, a variety of cell lines of the myeloid lineage have also been used including THP-1, KG-1, U-937, K-562 and HL-60.[130,137–140,149,150] In particular THP-1 and U-937 cell lines have been widely utilized in predicting the sensitization potential of chemical allergens based on Human Cell Line Activation Test (h-CLAT),[8,135] and myeloid U937 skin sensitization test (MUSST),[126] respectively. An inter-laboratory study assessing the ability of this test to distinguish sensitizers from non-sensitizers was also initiated (Table 2).

h-CLAT entails exposure of THP-1 cells to sensitizer or irritant for 48–72 h. Following this, the cells are tested for surface marker expression of CD54 and CD86. Inter-laboratory variation is included to identify a metric wherein if 2 out of 3 independent experiments show CD54>200% and CD86>150%, then the chemical is a sensitizer. Table 2 lists the number of chemicals that have been predicted as sensitizers using the h-CLAT or MUSST assays. While h-CLAT can predict sensitizers at a reasonable accuracy of 85% for 100 chemicals, MUSST assays predict sensitizers at accuracy of 85% for 99 chemicals.

However, these cell lines have certain limitations. For example, maturation of DCs derived from these cell lines is not necessarily correlative with increased antigen uptake and lymph node migratory capacity. Moreover, these cells do not undergo a DC-like differentiation pathway and hence are not necessarily good model systems to simulate the developmental process. Thus, although, these cell lines are available in potentially unlimited quantities, they may lack certain critical phenotypic characteristics necessary for assessing sensitization.

A promising cell line is the Mutz3 cell line, which was derived from the peripheral blood of a patient with acute myelomonocytic leukemia and exhibits morphological and phenotypical characteristics of monocytes. [22,84,128,137–140] These cells can be induced by cytokines and growth factors to differentiate into LC and DC lineages with close resemblance, from a functional marker expression profile, to epidermal LC[25,51,158] and DCs.[89] These studies suggest that Mutz3 cells is a reliable cell source for generating both LCs and DCs.[70] More

importantly, Mutz3 cells can present antigen and can migrate into lymph channels in response to CCL19 chemokine.[117] In response to strong sensitizers such as dinitrochlorobenzene (DNCB), 2,4,6-trinitrochlorobenzene (TNCB) and moderate sensitizers such as eugenol, increased expression of HLADR, CD54 and CD86 has been stimulated on Mutz3 cells[9] and Mutz3 derived LCs.[119]

In addition to maturation and migration of DCs, the activation and clonal expansion of allergen responsive T-lymphocytes represent other downstream events in the demonstration of skin sensitization, and are attractive targets for development of alternative approaches. Past studies have employed co-culture of activated LCs or DCs with allogeneic naïve T-cells, and then measured proliferation by incorporation of radioactive tritium[104] or measurement of IFNγ.[94] An extensive review of T cell based priming assays is elucidated in the literature.[94]

Further details of cell based priming assays elucidated in the literature[39,160] conclude that IL-8,[116,126] CD86,[119,153,165,168,169] and P38 MAP kinase[6,52,81,95–97,102,103,114,154] are the three commonly used metrics to distinguish sensitizer from irritant in various DC primary cells and cell line models.

The testing of chemotactic migration of cells in response to sensitizer or irritant in 2D model systems has not been reviewed previously in the literature. The effect of these chemicals on migration of cells in response to chemokines is a key determinant in the subsequent activation of T cells as part of the contact hypersensitivity phenomena. Thus, there have been few reports in the literature regarding migration as a metric to distinguish sensitizer from irritant treated LCs in response to a chemokine gradient.

There is at least one report that the number of LCs migrating post-sensitization *in vivo* is significantly higher for sensitizer vs. irritant treated cells irrespective of T cell number in the lymph node compartment.[63] Recent work also highlights the effect of chemokines on migration of cells *in vitro*. To simulate hapten-induced migration of LCs from the epidermis to the dermis, a dual chamber experiment was designed. This migration depends on 2 fibroblast-derived chemokines, i.e. CXCL12 and CCL5. Pre-treatment of fluorescently labeled (CSFE) MUTZ-LC (upper compartment) with sensitizers, but not with irritants, induces the expression of a CXCL12 receptor and, hence, enhances migration towards CXCL12 (lower compartment). For every chemical, the index of migration directed towards CXCL12 vs. that directed towards CCL5 can be determined. An index of CXCL12:CCL5>1, therefore, indicates sensitizers, and values <1 indicate non-sensitizers.[119]

Literature evidence also shows the utilization of CD34+ mononuclear peripheral cells for migration assessment in response to CCL19. The literature evidence for CCL19 based migration in presence of $NiSO_4$ or DNCB reveals significant migration thus confirming that sensitized mononuclear peripheral cells undergo migration in response to strong and extreme sensitizers.[20] From a mechanistic perspective, there is also literature evidence of PGE_2, a lipid mediator and its effect on migration of CCR7 positive cells in response to a chemokine.[141] Thus, based on recent literature evidence, the identification of migration of

DCs in response to chemokines could be a determinant metric to distinguish sensitizers from irritants.

However, the lack of sufficient data on a diverse chemical data-set and the lack of extensive validation in terms of inter and intra-laboratory variability reduces enthusiasm towards this concept for accurate prediction of skin sensitization of chemicals unless rigorously pursued.

In the studies mentioned above, monocultures of LCs and DCs were directly activated by the allergens. For example one group assessed the effect of a panel of known human contact allergens (1-fluoro-2,4-dinitrobenzene, DNFB; paraphenylenediamine PPD; methylchloroisothiazolinone/methylisothiazolinone, CMIT), as well as the skin irritant benzalkonium chloride and of the mitogen phorbol myristate acetate (PMA). It was found that a distinct response, as ascertained through Il-1B mRNA measurements, was elicited through treatment with each of the compounds.[121] An interesting finding, though, was that there is a potential for donor lot-to-lot variation, which is one important when assessing the sensitization potential of new compounds and formulations, and thus being able to tease out population dynamics through the use of multiple lots is crucial. Another interesting finding was that the degree of cellular response in this study was not directly related to the severity of the sensitizer. Thus, in these cases, the allergens had no exposure to skin cells which is an important prerequisite for LC and DC activation,[161] and also important transport dynamics were available within a purely single cell type based assay. Towards this end, one recent study describes a loose fit co-culture model of activated keratinocytes and DCs. This model is composed of a single layer of human non-differentiating keratinocytes and of allogeneic floating monocytes, which are co-cultured in presence of IL-4, GMCSF and TGF-β. This model uses the expression of CD86 as an endpoint, and was able to predict sensitization potential of both weak and strong sensitizers.[161]

Finally, none of the studies mentioned above takes into account the role that the skin structure plays in the sensitization process. Moreover, the effect of the sensitizers in direct monoculture with DCs is variable and not sufficient to induce significant enhanced expression of phenotypical markers.[110] Therefore, there would seem to be a real need for the development of suitable *in vitro* systems that incorporate human skin equivalents in co-culture with human DCs that can mimic the *in vivo* ACD phenomena more accurately.

3D Skin Tissue Based Systems

While the 2D cell models described above may be useful tools in certain studies, they certainly do not accurately simulate the *in vivo* condition, as skin is a complex three dimensional tissue with considerable barrier and metabolic function. To address this limitation, DCs derived from blood have been successfully integrated into a 3D cell culture model and used this model to predict sensitization in response to known allergens and UV radiation, where the end point metric was measurement of IL1β and CD86 mRNA, and the reduction in LCs, presumably due to LC emigration in response to the allergens.[46] These 3D skin models offer the benefit of testing whether a particular chemical can overcome the stratum corneum barrier and penetrate into the viable epidermis below. This step is particularly important for classification because potency prediction based solely on 2D cell based models may not be an accurate depiction of bioavailability of the compound *in vivo*.

This was found to be the case where an *in vitro* assay measuring CD86 expression on DCs showed very different sensitizing properties of *p*-toluylenediamine (PTD) and hydroxyethyl-*p*-phenylenediamine (HE-PPD) when compared to *in vivo* data.[2] This discrepancy could be due to the differential skin penetration and haptenation profiles of these chemicals yielding different end-point sensitization outcomes. Metabolic activity of skin has been tested recently based on cytochrome P450 enzyme activity in presence of pro-haptens.[10,61] Another advantage of 3D models is the ability to test chemicals that have low aqueous solubility by topical application. In the following paragraphs, we describe the different skin models and their utilization in simulating the sensitization phenomena.

Three broad classes of 3D skin models include excised skin, engineered epidermal models, and full thickness skin equivalents (Fig. 4).

Freshly excised human skin offers many advantages over engineered models since it retains a more *in vivo* like barrier and also features the full complement of accessory structures and cell types such as keratinocytes, fibroblasts, melanocytes, LCs and endothelial cells.[124] Contrary to expectations, human skin explant studies show that there is a higher percentage of CD1a+ CD83+ LCs post migration for toxic concentration of irritant vs. non-toxic concentration of sensitizer in an *in vitro* 3D model system.[66,88] It seems that all cells migrating in response to chemokine are CCR7+ in general. Due to the relatively sparse LCs and DCs in the epidermal and dermal layers, monitoring and quantifying migration rates of LCs and DCs in response to sensitizer remains challenging. The other major limitation of this model is large donor variability due to differences in lipid composition, skin thickness, hydration, age, sex and location of harvest. [42,82,86,123] Furthermore, due to its relatively short shelf life, it is not a practical model for allergen testing.

Engineered epidermal models are an attractive alternative since the epidermis is the site of entry and the location where hapten binding occurs. Keratinocytes in the epidermis also play a critical role in initiating and modulating the inflammatory reaction during sensitization. There are several commercially available epidermal equivalents that have been used for testing a variety of chemicals. Of these, three particular models (Episkin, Epiderm and SkinEthic's Re-constructed Human Epidermis (RHE)) were recently accepted by the European Commission for skin irritation testing.

SkinEthic's Episkin model consists of type I collagen matrix, representing the dermis, surfaced with a film of type IV human collagen upon which differentiated second passage keratinocytes (derived from mammary/abdominal samples obtained from healthy consenting donors during plastic surgery) are layered. [111] This skin model has been widely used in the literature for screening a wide variety of cosmetic formulations and irritants using metrics such as IL1α, IL-8 secretion and MTT for viability.[31,136] The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. A main application allows assessing the viability and the proliferation of cells. It can also be used to determine effect of cytotoxic chemicals on stimulation or inhibition of cell viability and growth. Epiderm is composed of keratinocytes from a single donor cultured to form a multi-layered, highly differentiated model of the human epidermis on permeable culture inserts. Like Episkin, this model was also widely used in screening for a

variety of sensitizers, and irritants using metrics such as MTT conversion for viability and testing for various secretion markers such as IL1α, IL-8, IL-1ra and PGE_2 [47]; SkinEthic's RHE model is described as an epidermis reconstructed by air lifted culture of normal human keratinocytes for 17 days in chemically defined medium on inert poly-carbonate filters. The RHE model has been shown to release IL-1α and IL-8 in a manner that corresponds with the MTT assay to distinguish between irritants and sensitizers.[30,125]

The inclusion of fibroblasts in the skin model may be important because fibroblasts and keratinocytes have been shown to synergistically perform during irritation and sensitization to produce secondary cytokines and chemokines such as IL-6, GM-CSF, CXCL12, CCL2 and CCL5.[18,29,117,118] Furthermore, there is evidence that fibroblasts aid in the maturation of dermal DCs via direct cell contact and through soluble factors such as TNF-α.[134] Thus, since full thickness models contain keratinocytes and fibroblasts, these models possess properties that reflect more physiologically relevant conditions. There are several commercially available full thickness models such as TestSkin, Apligraf, Skin,[34] AST-2000 and RealSkin. TestSkin and Apligraf are both products from Organogenesis Inc. and consist of keratinocytes on top of a fibroblast populated bovine collagen type I matrix. Both products have been used to screen a range of chemicals such as sodium dodecyl sulfate (SDS), Vaseline and calcipotriol, where MTT was used for viability testing and secretion of PGE_2, IL1α and IL-8 were assessed.[115] However, Apligraf is commonly used in the context of wound healing and may not be an ideal candidate for the purpose of irritant and sensitizer screening. Skin[34] is a skin equivalent from Advanced Tissue Sciences and consists of human keratinocytes and dermal fibroblasts co-cultured on a nylon mesh. A variety of surfactants and cosmetic formulations were tested on Skin[34] where MTT conversion was assessed in addition to measuring IL1α, IL1ra, IL6, IL-8, GM-CSF and PGE_2 levels.[98] AST 2000 is a full thickness model from Cell Systems where keratinocytes are seeded on top of a dermal equivalent. This model was used to test sensitizers oxazolone, 1-fluoro-2,4-dinitrobenzene (DNFB) and irritants SDS and TritonX-100, where p38 and JNK1/2 were activated by phosphorylation exclusively with sensitizer treated conditions and ERK1/2 was only activated by irritant conditions.[78] RealSkin is a full thickness model from SkinEthic consisting of a dermal equivalent where a lattice with acid-soluble collagen and normal human adult fibroblasts is overlaid by a stratified, well differentiated epidermis derived from normal human adult keratinocytes. Since this is a newer product, it has not been studied extensively for irritation or sensitization. However, mRNA expression levels for metabolic enzymes in RealSkin were found to be comparable to that of *in vivo* human skin.[92]

Overall, in terms of ease of availability, minimal batch to batch variability, ease of investigating functional outputs and ability to recapitulate *in vivo* like conditions, the engineered full thickness skin models are convenient and practical platforms for sensitization of DCs in co-culture model systems.

Once an engineered skin model is identified, sensitization studies can be conducted using co-cultures of skin and DCs as shown in Fig. 5. For example, skin can be mounted in a transwell, while DCs reside in the bottom chamber. In a typical experiment, a chemical is applied topically onto skin, generally via some type of absorbent material placed on top of the skin. After the chemical has penetrated into the skin and activated the DCs in the bottom

chamber, a number of assays can be performed for assessing allergen potential of the chemical. The various assays include measuring chemotactic migration capacity of DCs, assessing expression of surface markers, gene expression patterns and relevant signal transduction pathways of activated DCs as indicators of DC maturation and DC induced stimulation of T-cell activation.

Microfabricated Platforms

As described above, all the candidate *in vitro* assays typically include as their endpoint, only one or two of the hallmark events that occur during the demonstration of skin sensitization. Although consideration of one endpoint in isolation may provide some information regarding the sensitization potential of a chemical, they do not achieve the same predictive accuracy as *in vivo* methods such as LLNA (Table 2) and do not provide mechanistic insights into the contact hypersensitivity phenomena. It is likely that the development of a platform that successfully brings together all the inductive events involved in skin allergy will potentially provide investigators with a tool for studying the mechanistic aspects of these complex phenomena as well as prediction of sensitizers using this “LLNA-like” assay system. The disciplines of microscale engineering in conjunction with tissue engineering can potentially provide such a platform and revolutionize the *in vitro* allergy test field.

Integration of microscale technologies with cell and tissue culture is emerging as a powerful approach for creating advanced cell culture models.[41,100] Microtechnology, originally developed in the semiconductor industry, provided the basis for fabrication of microdevices with a characteristic feature size ranging from microns to millimeters. Initial approaches for fabrication relied on photolithography and etching based techniques.[62] With advances in soft lithography, fabrication of microdevices in elastomeric materials such as poly (dimethylsiloxane) became relatively easier.[166] The ease of fabrication in combination with other desirable properties such as gas permeability, optical transparency, and compatibility with biological assays further paved the way for utilizing elastomer based microdevices for cell culture. There are several advantages in utilizing microdevices for cell culture. Microfluidics facilitates exquisite spatial[152] and temporal [77] control over the cellular microenvironment, which were previously unattainable using conventional cell culture methods. In addition, scaling down to the micron level permits significant reagent and raw material saving, and has the added benefit of the possibility of evaluating responses at a single cell level.[132] Microdevices can also be used to develop interconnected cell culture chambers that mimic the interconnectivity found *in vivo*, thus providing further relevance and presumed predictability.[26,74,93,113,144] For example, multi-tissue device have been developed that allow for the assessment of drug metabolism (clearance), as well as volume of distribution for new chemical entities. These chips have the potential to act as a surrogate for animal testing, in that they can be integrated with human cell types thereby reducing the necessity for animal models, or even animal derived cell types.[26,93,113] In addition to the advantage of miniaturized cell and multi-tissue culture models, chemotaxis (another cell and tissue level process important in allergic immune response) has been successfully adapted to the microfluidic format.[60,65,72,90] In comparison to traditional methods, microfluidics can reproduce *in vivo* like channel sizes, and the establishment of more precise spatially and temporally stable chemokine gradients.[90] Fast liquid switching capability provided by

microfluidics further facilitates realization of user-defined chemokine gradients.[72] Additionally, controlling and quantifying gradients at the cellular level together with microscopic monitoring of the movement of single cells, offers the potential to establish quantitative correlations between cell migration and response to a chemokine gradient at a single cell level.

Given the flexibility of microdevices in designing interconnected tissue compartments which can integrate chemotaxis, our laboratory begun developing a microfabricated platform that has the potential to essentially capture the majority of the key steps involved in the sensitizer-induced immune response. As shown in Fig. 6, a microdevice can include both skin and lymph node compartments connected via microchannels. [167] The skin compartment can contain the engineered skin model with DCs either integrated in the skin model or residing directly underneath the skin. In response to an allergen, DCs in the skin compartment can become activated by sensitizers, leading to their migration to the lymph node compartment where they can activate T cells.[79] The microfabricated system has the potential to mimic the essential elements of LLNA, including topical application of chemical on skin, intercellular communication between skin resident cells, maturation and chemotactic migration of DCs, and T cell activation by DCs. This system offers a comprehensive set of *in vitro* assays with the collective potential of developing into an alternative to LLNA for animal free testing of chemicals.[142] However, this assay system, due to its nascent technological development status, needs validation.

HUMAN REPEATED INSULIN PATCH TEST (HRIPT)

HRIPT has a 50 year history and was first developed in 1944 by Schwartz and Peck. Quantitative risk assessment of sensitizers utilizes HRIPT in combination with LLNA[50] though recent literature mentions the ethical concerns associated with HRIPT.[159] Human tests for assessment of chemicals as sensitizers is ethical based on written informed consent of patients and is conducive as a comparison of the skin sensitization challenge to improve robustness of historical or novel control approaches.[12] Detail protocols and factors implicated in HRIPT have been cited in the literature. [99] There have also been recent reports on the genetics of contact allergy and its relationship to polysensitization.[157] The cumbersome and lengthy procedure to test chemicals in human volunteers and the ethical concerns associated with the protocol dampens enthusiasm towards the large scale implementation of this method for skin sensitization studies. However, for validation of compounds especially of complex molecular structure, unknown mechanistic applicability domains and pro-haptens, the human patch test can be a fairly robust and conclusive endpoint assay.

INTEGRATED TESTING STRATEGIES

As described above, all the candidate assays do not achieve the same predictive accuracy as *in vivo* methods such as LLNA (Table 2). Integrated testing systems comprising of a combination of the above discussed approaches could be the most optimal solution for validation of chemicals categorized as sensitizer/non-sensitizer based on the LLNA, human or GPMT datasets [69,107] and/or prediction of new chemicals.

Examples of multi-tier systems from the literature are described:

- Incorporation of a step-wise QSAR model domain based on different descriptors could work for predicting sensitization potential of chemicals.[37]
- Another example is inclusion of compounds that have failed positive testing using Natsch and Emter method[108] as sensitizer, though the chemical is identified as a sensitizer using the QSAR based models (e.g.: aldehydes and ketones). The Natsch and Emter methodology is an elaborate and very promising method that is based on antioxidant response gene expression changes (Keap1) in presence of sensitizer for AREc32 cell line (CXR Biosciences) that uniquely links metabolic mechanism of cells to sensitizer-protein binding. Also, currently, CXR Biosciences is working with collaborators to introduce adenoviral vectors that lead to expression of multiple cytochrome P450 isozymes in response to specific pro-haptens in these cell lines. Recently, the Natsch approach has been integrated with peptide reactivity assay and TIMES-SS platform with 88% prediction for 116 chemicals in comparison to the LLNA database (Table 2). This set of chemical classification is further scored by each tier of the system according to proposed guidelines.[69]
- In a recent study an integrated approach that combines in chemico coupling (peptide-binding) assay) with T-cell activation was described.[36] The chemicals that form adduct with the skin relevant protein such as human serum albumin, or directly modified cellular proteins in dendritic cells induced T cell response as estimated by measuring T cell proliferation and interferon gamma production. The two step approach further underscored the difficulty associated with interpretation of results obtained in single assay in isolation. For example direct treatment of immature DCs with the sensitizer DNCB failed to generate robust T cell response. The failed T cell response was explained based on the peptide binding assay that revealed inefficient modification of peptide by DNCB.

As described above, the major limitations of integrated systems are the conflicting results in the multitier approach and they have not been extensively tested for large chemical data-sets.

To develop an optimal integrated testing platform, it is imperative to firstly generate a formal list of chemicals that cover the entire range of mechanistic reaction domains, complex chemistry and metabolic transformation capacity.[43,58] Further validation of each tier of the integrated system utilizing chemical data-sets such as LLNA and categorically scoring chemicals based on sensitization potency is essential.[69] The development of a pre-validated multi-tier integrated system with multiple biological end-points can then be utilized for predicting sensitization potency of unknown chemicals with reasonable confidence.

SUMMARY

In the current paper, we have discussed key technologies that can potentially replace the animal-based gold standards, such as the LLNA, for assessing sensitization potential of chemicals. These *in vitro* and *in silico* approaches described above offer alternatives that can

potentially limit or even eliminate the use of animal models for testing of sensitizers. While the *in silico* QSAR based models have been extensively tested for a large chemical data-set, the *in vitro* models, such as the DPRA and 2D based models, while are promising, however, do lack extensive validation. The 3D based models and the microfluidic platform are interesting solutions to the underlying problem of capturing the complexity of ACD associated events *in vitro* but they lack validation.

Overall, despite their utility, these entire model systems display some inherent limitations as summarized in Table 1. Also, as shown in Table 2, there is some percentage of chemicals that cannot be predicted accurately as sensitizers or non-sensitizers in comparison to the animal tests derived experimental data-sets.

At one end, there is a requirement for developing novel *in vitro* techniques that target specific chemicals of complex cases based on reaction mechanistic domains, structural alerts and difficult chemistry.[58] At the other end, there is an urgent need to utilize already existing robust approaches and novel *in vitro* techniques under development to be implemented in an integrated platform based on existing experimental data to validate set of chemicals.

ABBREVIATIONS

ACD	Allergic Contact Dermatitis
BBB	Blood Brain Barrier
BgVV	Bundesinstitut für Gesundheitlichen Verbraucherschutz Und Veterinärmedizin
DC	Dendritic Cells
DEREK	Deductive Estimation of Risk from Existing Knowledge
DNCB	DiNitroChloroBenzene
DNFB	1-Fluoro-2,4-dinitrobenzene
DPRA	Direct Peptide Reactivity Assay
GPMT	Guinea Pig Maximization Test
h-CLAT	human Cell Line Activation Test
HE-PPD	Hydroxyethyl-*p*-phenylenediamine
HRIPT	Human Repeated Insulin Patch Test
HRP/HP	Horseradish Peroxidase/Hydrogen Peroxide
ITS	Integrated Testing Strategies
LC	Langerhans Cells
LR	Linear Regression
LLNA	Local Lymph Node Assay
MUSST	Myeloid U937 Skin Sensitisation Test

MULTICASE	Multiple Computer Automated Structure Evaluation
NCHS	National Center for Health Statistics
OECD	Organization for Economic Cooperation and Development
PCA	Principal Component Analysis
PTD	*p*-toluylenediamine
QSAR	Quantitative Structure Activity Relationship
QSPR	Quantitative Structure Property Relationship
REACh	Registration, Evaluation, Authorization of Chemicals
SAR	Structure Activity Relationship
TIMES-SS	TImes MEtabolism Simulator platform used for predicting Skin Sensitization
TNCB	2,4,6-Trinitrochlorobenzene
TOPKAT	Toxicity Prediction by Komputer Assisted Technology

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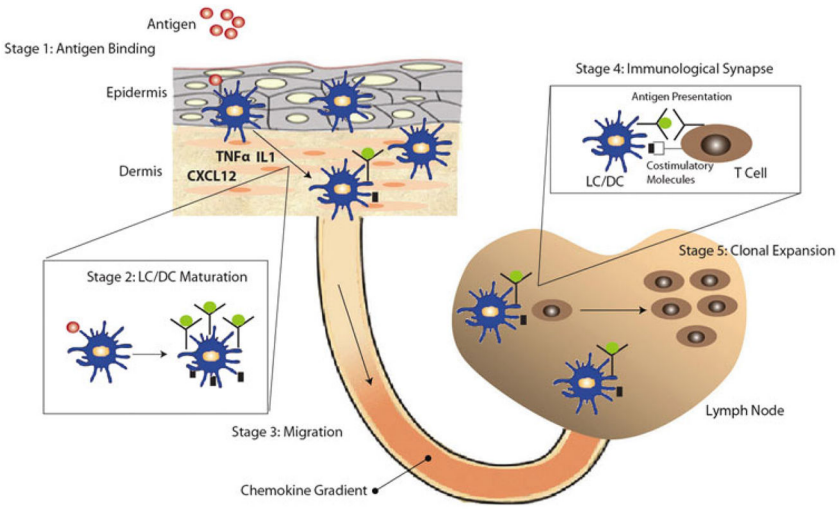

FIGURE 1.

Schematic illustrating the complex cascade of events associated with the immune response to an allergen. The exposure to an allergen leads to the maturation of LCs/DCs and migration of LC/DC to the localized lymph node. The activation and migration of DCs is regulated by multiple factors including chemokines and cytokines such as TNFα, IL1, and CXCL12 secreted by various resident skin cells. Allergen induced maturation results in the LC/DCs acquiring antigen and up regulation of co-stimulatory molecules, and chemokine receptors such as CCR7. The migration of LC/DC is believed to be regulated by chemokine gradient of CCL19 and/orCCL21 across the lymphatic vessel. The migrated LC/DC forms an immunological synapse with the T cells, which leads to the activation of T cells and their clonal expansion.

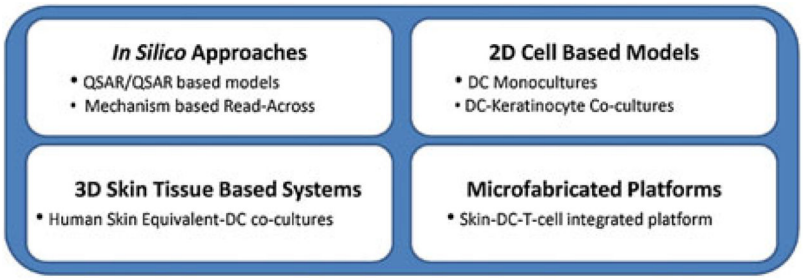

FIGURE 2.

Overview of non-animal alternative methods for sensitization testing. There are four classes of non-animal alternative methods. (1) *In silico* approaches. (2) cell based models. (3) 3D skin tissue based models and (4) an integrated microfluidic platform.

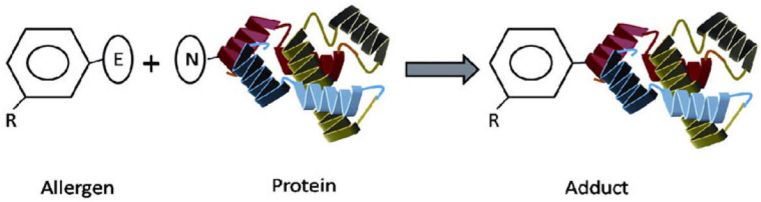

FIGURE 3.

Schematic of direct peptide reactivity assay. The assay is based on evaluating covalent bond formation between nucleophilic protein and electrophilic allergen. The reaction is assessed by measuring either nucleophile depletion or adduct formation.

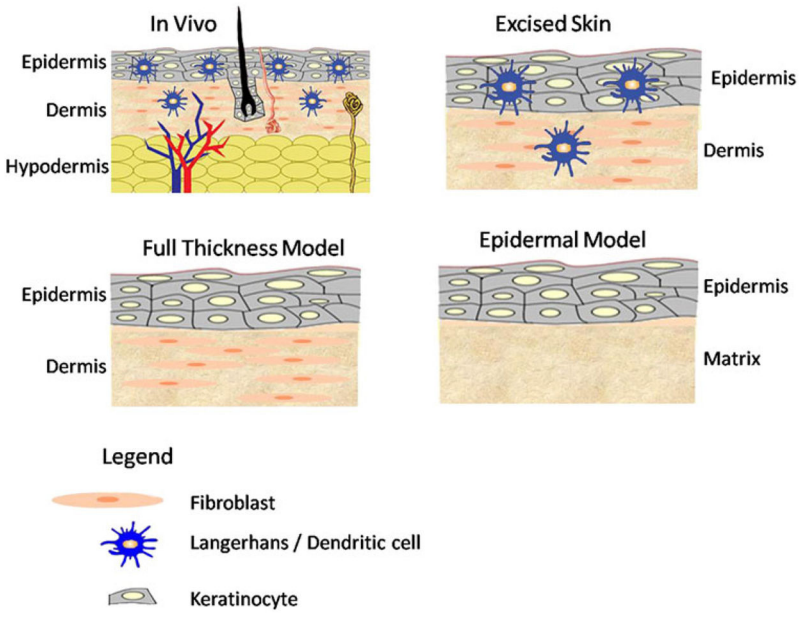

FIGURE 4.

Schematic of various skin models that can be potentially utilized for sensitization experiments. The excised skin model consists of the full complement of cells ranging from epidermal LCs, dermal DCs, keratinocytes, and fibroblasts. The engineered skin constructs are epidermal and full thickness models. The epidermal model is comprised of keratinocytes, while the full thickness model also includes fibroblasts.

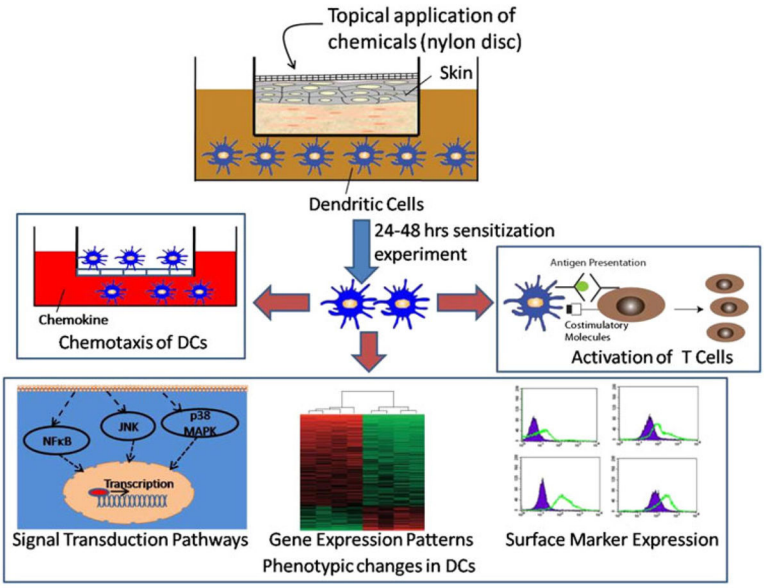

FIGURE 5.

Schematic of a typical sensitization experiment. A chemical is applied topically to the skin, which is in co-culture with the DCs. After the chemical has penetrated into the skin and activated the DCs, various functional assays are preformed that include measuring chemotactic migration capacity of DCs, assessing expression of surface markers, gene expression patterns and signal transduction pathways of activated DCs as indicators of dendritic cell maturation, and DC induced stimulation of T-cell activation.

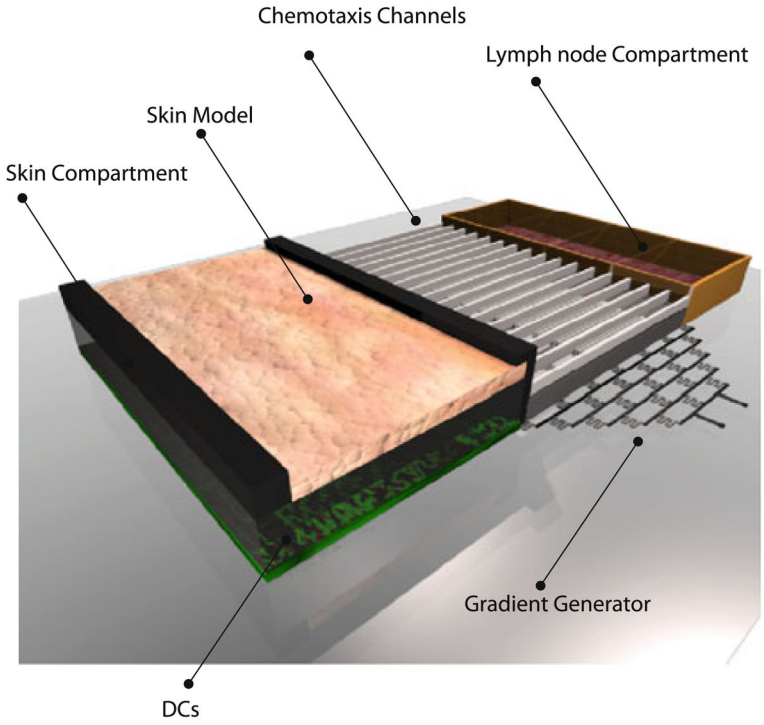

FIGURE 6.

Schematic of microdevice that can potentially be used for performing the LLNA on chip. The microdevice consists of the skin and the lymph node compartments connected via microchannels. The skin compartment can contain the engineered skin model with DCs either integrated in the model or residing directly underneath the skin. In response to an allergen, DCs in the skin compartment can get activated leading to their migration to the lymph node compartment where they can activate the T cells.

TABLE 1

Comparison of non-animal alternative methods for assessing sensitization potential of chemicals.

Model systems	Description	Advantages	Disadvantages
In silico approaches			
DEREK	Uses structural alerts for sensitization and physiochemical properties of chemicals and provides degree of confidence of chemical as sensitizer	User-friendly and takes into account type of animal model used for sensitizer	Does not take into account electrophilicity of chemicals
TOPKAT	Uses structural alerts present in prediction space and provides probability score of chemical as sensitizer	Takes into account large scale chemical datasets	Does not take into account electrophilicity of chemicals
TIMES-SS	Incorporates skin metabolism and interaction of chemical with reactive proteins	Incorporates skin metabolism of pro-haptens for assessing sensitization potential	Requires testing large data-sets for validation; does not take into account reaction chemistry pathways
Mechanism Based Read-Across	Incorporates reaction between electrophilic sensitizer and nucleophilic skin peptides	Incorporates electrophilicity index of chemical for sensitizer classification purposes	Requires *a priori* EC3 data
In vitro approaches			
Peptide binding	Chemical reacts with proteins containing Cys and Lys forming stable covalent bonds	High-throughput, simple assay	Not accurate with weak sensitizers and pro-haptens
2D cell based models	Incorporates keratinocytes, primary DCs and DC cell lines (Mutz-3, THP-1, U937, KG-1, K-562, HL-60)	Incorporates dendritic cell-sensitizer interaction	Lacking skin tissue
3D Skin tissue based models	Epidermal equivalents, full thickness models, excised skin in co-culture with DCs	Topical application, Incorporates skin-DC interaction	Lacks dynamic interaction between skin, DC and T cells
Microfluidic platform	Microfabricated system integrating skin and lymph node tissues	Integrated approach; reduced cell and reagents requirements	Requires validation; relatively nascent technology

TABLE 2

In silico QSAR and *in vitro* models for prediction of sensitization capacity of chemicals.

Assay	Significant features	Number of compounds	% Prediction of sensitizer	% Prediction of non-sensitizer	Model system	References
In silico						
LDA QSAR	14 parameter model; HOMO-LUMO energy gap; Shannon index; discriminant analysis	259	76.5	87.9	GPMT	[33,35]
Neural network QSAR	14 parameter model; HOMO-LUMO energy gap; Shannon index	259	83.2	94.3	GPMT	[35]
SAR	Skin metabolism; electrophilic interactions; molecular structure and reactivity; steric effects	106	Qualitative	Qualitative	LLNA	[7]
QSAR	2D or 3D molecular r structure descriptors; topological indices; autocorrelation functions; logical regression	54	72	93	LLNA	[49]
DEREK, TOPKAT and linear regression	TOPKAT is GPMT based; DEREK and LR model is based on LLNA and GPMT	178	82.9, 73.3, 87.6	82.9, 73.3, 87.6	GPMT	[48]
DEREK and linear regression	DEREK and LR model is based on LLNA and GPMT	178	73, 83.2	73, 83.2	LLNA	[48]
TIMES-SS	Skin metabolism model; COREPA 3D-QSAR	634	65	72	LLNA, GPMT, BgVV	[37]
4D-QSAR	Two-2-state (sensitizer, non-sensitizer) categorical model; 3D structure of molecules	196	91.5	93.9	LLNA	[91]
QSPR	Literature and structural descriptors	358, 307, 251	90, 95, 90	ND	LLNA, GPMT, BgVV	[59]
TOPSMODE-QSAR	Tonnage amount, cluster analysis; prediction of hair dye substances; only 10% of chemicals were pre-tested using LLNA and/or human tests	229	Qualitative	Qualitative	LLNA and human tests	[146]
Physiolab	Epidermal inflammation; LC maturation markers; Ag specific T cell proliferation	8	100	0		Entelos
DEREK/METEOR	Expert system/metabolic fate predictor	Not disclosed	Not disclosed	Not disclosed	Not disclosed	LHASA limited
TOXHUNTER	Genomic, proteomic and metabolomic compound signatures	Not disclosed	Not disclosed	Not disclosed	Not disclosed	Genego
BioEpisteme	2D or 3D molecular descriptors	Not disclosed	Not disclosed	Not disclosed	Not disclosed	Prous Institute
In chemico						
GSH/Cys/Lys PRA	Six models; different ratios of GSH, Cys, or Lys to chemicals; recursive partitioning model based tree classification to compare LLNA EC3	82	89	89	LLNA	[58]
DPRA	Different ratios of GSH, Cys, or Lys to chemicals; GSH (1:100), Lys (1:50), Cys (1:10), His(1:50)	38	66, 67, 84, 36	66, 67, 84, 36	LLNA	[57]

Assay	Significant features	Number of compounds	% Prediction of sensitizer	% Prediction of non-sensitizer	Model system	References
DPRA	Horseradish peroxidase and hydrogen peroxide assay; highly sensitive HPLC tandem mass spectrometry	32	100	Not Determined	LLNA	[56]
Cell based						
h-CLAT	3 rounds of trial for inter-laboratory [5 labs] consistency testing; CD54 and CD86 expression of THP-1 sensitized cells	27	84	84	LLNA	[135]
h-CLAT	CD54 and CD86 expression of THP-1 sensitized cells	100	85	85	LLNA	[8]
MUSST	CD86 expression of U-937 sensitized cells	99	85	Not determined	Human data	[1]
Keratinosens	Trial for inter-laboratory [5 labs] consistency testing; ARE expression of HaCat sensitized cells	28	91	91	LLNA	[5]
ARE luciferase assay	ARE gene expression in AREc32 cells; seeded in 96-well plates at a density of 50,000 cells	102	83	83	LLNA	[108]
Integrated testing strategy						
Peptide/ARE/TIMES-SS	ARE expression of HaCat sensitized cells, TIMES-SS and peptide reactivity; data integration from multiple systems using regression analysis	116	87.9	87.9	LLNA	[109]