Session I-11: Safety testing for skin sensitization hazards: Recent Three Rs advances

Session I-11: Oral presentations

I-11-626

Alternative approaches for the evaluation of skin sensitisation and their use for quantitative risk assessment of cosmetic ingredients

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In 2010 a panel of scientific experts was tasked with assessing the availability of alternative methods to animal testing for five toxicological areas, including skin sensitization, in view of the full marketing ban anticipated in 2013 for cosmetic products and ingredients tested on animals in Europe. In the absence of validated alternative methods, predictive testing for skin sensitisation still relies on the use of animals. The key mechanisms at the basis of the induction of skin sensitisation are rather complex but relatively well understood, and this knowledge is being exploited in the development of mechanistically-based non-animal test methods primarily designed for hazard identification. However, skin sensitization risk assessment decisions require not only hazard identification, but also sensitizer potency information to allow a safe level of human exposure to be predicted. It is proposed that a range of mechanistically based non-animal test methods would be necessary to yield an alternative measure of skin sensitiser potency. However, at present it is not possible to predict which combinations of non-animal information will be needed before risk assessment decisions could be exclusively based on non-animal testing data with sufficient confidence for the vast majority of cosmetic product exposure scenarios. The expert group concluded that, by 2013, full replacement of animal methods will not be available for skin sensitising potency assessment. The most positive view of timing for full replacement is another 7-9 years (2017-2019), although it is expected that the scientific ability to inform skin sensitization decisions without animal test data for some ingredients and exposure scenarios should be feasible ahead of 2017-2019.

I-11-051

The COLIPA research and method development program for identifying and characterizing skin sensitizers without animal testing

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At present, animal test methods (such as the mouse local lymph node assay) are required to characterize the potential for new chemicals to induce skin allergy. However there are currently several large research programs ongoing that aim to deliver new non-animal test methods for skin sensitization. COLIPA, the European Cosmetics Industry trade association, intensively participates in this international research effort through continuous funding of projects to explore the processes governing the induction of skin sensitization and the development of new methods incorporating the acquired knowledge. Our ongoing research portfolio (9 different research and method development projects investigating bioavailability, chemical reactivity, protein/peptide binding, skin metabolism, dendritic cell activation and migration mechanisms, T cell proliferation and multi-cell type interactions) continues to provide new insights into the biological processes driving skin sensitization and has already led to the successful development of three *in vitro* test methods for the detection of potential sensitizers: the Direct Peptide Reactivity Assay (DPRA), the human Cell Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitization Test (MUSST). These tests are currently at the ECVAM pre-validation stage. In parallel, a focused evaluation of other available test methods as well as the use of all these methods for risk assessment purposes is being conducted. This comprehensive research and development program aims to define a toolbox of assays to be used in a risk assessment strategy capable of characterizing skin sensitizer potential and potency, the final goal being to perform this risk assessment without the need for animal testing.

I-11-651

Modeling for the molecular mechanisms of allergens on the innate immune synapse

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Because innate immune cells have been implicated in the immune mechanisms of allergic contact dermatitis, we hypothesized that ligands involved in the activation of natural killer or natural killer T-cells may be perturbed in keratinocytes (KC) as a part of their phenotypic changes associated with an allergen induced stress response. To test this hypothesis, we first studied human allergic contact dermatitis in positive patch tests to $NiS0_4$. ULBP-2 was up-regulated on KC *in situ* in ACD (gene expression and *in situ* staining). Studies of primary cultured human KC confirmed the *in vivo* observations regarding the effects of $NiCl_2$ on cultured human KC and ULBP-2 gene expression. Gene expression profiling of human KC revealed that $NiCl_2$ consistently up-regulated a small number of genes involved in innate immune synapse, including ULBP-2. Exposure of primary KC to $NiCl_2$ prior to incubation with highly enriched NK cells resulted in significantly enhanced cytotoxicity (^{51}Cr release), which could be blocked by ULBP-2 specific monoclonal antibody. The KC-derived cell line HaCat and the THP-1 cell line (monocyte lineage) also increased ULBP gene expression after exposure to nickel, indicating consistency of this phenomenon. Ongoing studies are examining other allergens in the training set of chemicals to determine how sensitive and specific the ULBP gene set will be in detected allergens and irritants *in vitro*.

I-11-125

Assessing the sensitization potential of compounds without animal testing

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The FP6 project Sens-it-iv (www.sens-it-iv.eu) has developed promising tests for the identification of skin and respiratory sensitizers. The human reconstituted skin test and the NCTC2544 test are currently tested as a tiered strategy, where the NCTC test identifies skin sensitizers (>99% accuracy) and the reconstituted skin test predicts potency in good correlation with the LLNA. The concordance of the tiered approach with the LLNA for skin sensitizers is 79% for potency ranking and 92% for classifying compounds into the correct potency groups (extreme, strong, moderate, and weak). A DC (dendritic cell) marker signature of 75 genes predicts skin sensitizers with 98% accuracy. In addition, information about the potency of chemicals is provided. Another DC-based test addresses the degree of maturation of DCs before and after exposure to chemicals using a chip coated with monoclonal antibodies against membrane markers. This test has the potential to become a medium through-put alternative to methods such as HCLAT and MUSST. Finally, a functional test assessing exposure driven DC migration has the potential of becoming a first post-screen test. To date, no misclassifications have been observed with this test. An *in vitro* T cell-priming test has been established. This test is the most complex assay of the tool box. Good predictivity has been demonstrated using proliferation as well as TNF-α and IFN-γ levels as read-outs. Thus, tools are available that (i) can identify skin sensitizers and (ii) are able to provide information about the potency of these sensitizers.

I-11-323

Towards the development of a BioMEMS-based microsystem to assess chemical sensitization: Allergy-on-a-Chip

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Skin sensitization remains a major environmental and occupational health hazard. Animal models have been used as the "gold standard" and method of choice for assessing chemical sensitization potential. However, a growing international drive and consensus for minimizing and/or eliminating animal usage have prompted the development of *in vitro* methods to assess chemical sensitization. We are in the process of developing a microfabricated platform that can capture the majority of the key steps involved in allergic contact sensitization. This "Allergy-on-a-Chip" microfluidics-based device integrates a reconstructed human skin compartment with a dendritic cell culture compartment. Intercellular communication is initiated through microfluidically-mediated signaling between the skin construct where the allergenic stimulus originates (via topical application of sensitizer) and an immune system compartment where the response to that stimulus occurs. The microfluidic device is designed to 1) activate dendritic cells following allergen diffusion and/or conversion by skin constructs, 2) allow sensitizer-activated dendritic cell migration via optimized chemokine gradients, and 3) ultimately induce sensitizer-mediated T cell activation. To date, we have fabricated a microdevice and quantified cell-responsive chemotaxis. Using this device, we have established an experimental system integrating a full thickness skin substitute and Mutz-3 cells that have been differentiated into immature Langerhans cells. In addition, we have successfully validated the efficacy of "on-chip" sensitization with a panel of sensitizers, and demonstrated quantitative dendritic cell activation metrics that can be used to distinguish irritants from sensitizers.

Session I-11: Poster presentations

I-11-083

Role of AU-rich element binding proteins in mRNA stability and potency of chemical allergens

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We previously demonstrated in the human promyelocytic cell line THP-1 that all allergens, including prohaptens, selectively induce a rapid p38α MAPK activation and IL-8 mRNA expression. While the prohapten isoeugenol fails to induce IL-8 release, all other allergens tested induced a dose-related release of this cytokine.

In the present study, we investigated whether this abnormal behavior of isoeugenol is regulated by AU-rich element (ARE) binding proteins, namely HuR and tristetraprolin (TTP). THP-1 cells were treated with isoeugenol and, for comparison, with the moderate contact allergen diethylmaleate (DEM), with the strong contact allergen DNCB, and with the irritant salicylic acid.

Data obtained provide evidence of a different regulation of IL-8 during contact allergen treatment. Distinct combination and regulation of the ARE binding proteins HuR and TTP following contact allergen exposure resulted in a different modulation of IL-8 mRNA half-life and release. Data shown demonstrated that increased expression of TTP results in destabilization of the IL-8 mRNA in THP-1 cells treated with isoeugenol, which can account for the lack of IL-8 release. In contrast, the strong allergen DNCB, failing to upregulate TTP while inducing HuR, resulted in longer IL-8 mRNA half-life and protein release. DEM induced TTP at a later time point; it did not induce HuR, resulting in increased IL-8 mRNA half-life compared to isoeugenol but shorter than DNCB. It is tempting to speculate that this different behavior of allergens may also occur *in vivo*, and may contribute to our understanding of allergen potency.

I-11-088

SenCeeTox®: a new *in vitro* method for predicting photosensitization

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The safety of over the counter (OTC) drugs, cosmetics and personal care products is an important part of product development. Standard tests for chemical sensitization have depended on animals. Amendment 7 of the European Cosmetics Directive requires the development of *in vitro* methods that replace animal usage. Although some *in vitro* approaches are under evaluation, SenCeeTox® is the only method designed to identify sensitization potential and provide a potency category. This assay relies on a concentration response of genes controlled by the antioxidant response element (ARE), cytotoxicity, direct reactivity, solubility, and dermal permeability to predict the sensitization potential. The aim of this study was to determine if SenCeeTox® can be used to identify photoallergens. Four known photo activated chemicals (oxybenzone, avobenzone, octisalate, and padimate-O) relevant to the OTC drug/cosmetic industry were selected. Glycerol, p-benzoquinone, and naproxen were used as negative controls, while ciprofloxacin and TSA were used as positive controls for photoactivation. Test compounds were prepared in DMSO and then diluted into PBS. The samples were divided into two groups, one exposed to 6 J/cm^2 UVA light, and one that remained in the dark. Following the light exposure, an aliquot was removed and evaluated for direct reactivity using glutathione (GSH) depletion. A second aliquot was mixed with culture medium and applied to a human keratinocyte (HaCaT) cell in 96-well plates. Following a 24 h exposure, cells were assessed for cytotoxicity (MTT) and ARE controlled gene expression (qRT-PCR). Analysis of cell viability, gene expression, and reactivity data indicates that SenCeeTox® can be used to identify photosensitization.

I-11-100

The Colipa research and method development program for identifying and characterizing skin sensitizers without animal testing

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At present, animal test methods (such as the mouse local lymph node assay) are required to characterize the potential for new chemicals to induce skin allergy. However there are currently several large research programs ongoing that aim to deliver new non-animal test methods for skin sensitization. Colipa, the European Cosmetics Industry trade association, intensively participates in this international research effort through continuous funding of projects to explore the processes governing the induction of skin sensitization and the development of new methods incorporating the acquired knowledge. Our ongoing research portfolio (9 different research and method development projects investigating bioavailability, chemical reactivity, protein/peptide binding, skin metabolism, dendritic cell activation and migration mechanisms, T cell proliferation and multi-cell type interactions) continues to provide new insights into the biological processes driving skin sensitization and has already led to the successful development of three *in vitro* test methods for the detection of potential sensitizers: the Direct Peptide Reactivity Assay (DPRA), the human Cell Line Activation Test (h-CLAT) and the Myeloid U937 Skin Sensitization Test (MUSST). These tests are currently at the ECVAM pre-validation stage. In parallel, a focused evaluation of other available test methods as well as the use of all these methods for risk assessment purposes is being conducted.

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I-11-111

Prediction of skin sensitization potential of preservatives using CD54 and/or CD86 on THP-1 cells

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Evaluation of skin sensitization potential is a major part of safety assessment of new ingredients in cosmetics and drugs to be applied topically. To evaluate the skin sensitization potential, animal test methods such as the Guinea Pig Maximization Test have been used. From the standpoint of animal welfare, the establishment of *in vitro* skin sensitization test methods is necessary. Evaluation of changes in cell surface marker expression induced in dendritic cells (DC) or DC-surrogate cell lines following exposure to contact allergens represents one approach for the development of non-animal test methods for skin sensitization. The aim of this study is to confirm the predictive potential of an *in vitro* test method for skin sensitization. The aim of this study was to optimize an *in vitro* skin sensitization test using THP-1 cells (monocytic leukemia cell line) with CD54 or CD86 expression markers. We evaluated 11 preservatives (e.g., 1,2 Hexanediol, Phenoxyethanol, MCI/MI) in various concentrations. By evaluating the expression patterns of these indicating markers, we could classify the chemicals as sensitizers or non-sensitizers. These data suggest that the THP-1 cells are a good model for screening for contact sensitizers.

I-11-112

Validation of a skin irritation study using a Japanese model; LabCyte EPI-MODEL24, additional study

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Based on the EpiSkin statement and its protocol, we created a program to validate the usefulness, relevance and reproducibility (including intra- and inter-laboratory variability and transferability) of a skin irritation study using a Japanese model (LabCyte EPI-MODEL24) from 2008-2009. From these data, a good transferability of the LabCyte EPI-MODEL24 and the original protocol was obtained. The skin irritation categories (Non-Irritant or Irritant) for 25 blinded chemicals using this model and protocol showed good inter-laboratory and good predictivity in each laboratory. However, OECD peer review panel indicated 1-bromohexane, category 2, was misclassified into the "No" category by five of the six laboratories, and recommended that the issue be solved. In accordance with this recommendation, J-TEC revised the protocol.

In this study, our goal was to re-evaluate the predictive capacity of the revised protocol using 20 blinded chemicals from the reference chemical list in the OECD Performance Standards.

Based on the reference list in the OECD Performance Standards, a catch-up validation of the LabCyte EPI-MODEL24 SIT by three labs was performed. The assay demonstrated high reliability within and between laboratories, and acceptable reliability of accuracy (75-84.2% overall accuracy, 90-100% overall sensitivity, and 60-70% overall specificity) on the MTT assay excluding 60% of specificity at one laboratory. Two of three laboratories are sufficient with acceptance criteria according to the OECD Performance Standards and the VMT considered that this assay had acceptable reliability of accuracy for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

I-11-116

Application of the TTC and weight of evidence for the safety assessment of botanicals: Calendula and Juniper

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Botanical materials are increasingly being used in personal care products. The safety evaluation of these poorly characterized complex mixtures is a challenge due to the high degree of variability in composition. The first step is to identify each constituent chemical and its concentration range through literature search, supplier information, and/or analytical testing. For each chemical, skin penetration estimates can be derived from molecular weight and LogP values. The maximized, probable systemic exposure can then be established utilizing the concentration of the ingredient in a product and its application rate. If a NOEL or NOAEL does not exist for a chemical constituent, TTC is used as the first step in the safety evaluation. Based on Cramer Class the associated TTC value is compared to the systemic exposure. If the TTC value is exceeded, it is determined if there is a published Possible Average Daily Intake (PADI) for the chemical ("comparative approach"). If there is no known allowable daily intake or if systemic exposure exceeds this value, then a margin of safety must be estimated for the chemical. In order to estimate a margin of safety, a chemical of similar structure with a known NOEL or NOAEL can be used as "read across" surrogate (Chemical grouping approach). The MOS is then determined by dividing the NOEL or NOAEL by the systemic exposure and then applying the standard toxicological safety factors. A combined "weight of evidence" approach is very useful when evaluating such materials that tend to concentrate plant or oil components.

I-11-123

Acute dermal toxicity using the OECD TG 404 integrated testing strategy combining the use of the EpiSkin test methods

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Identification of corrosive and skin irritant chemicals was based, for regulatory purposes, on their ability to produce irreversible or reversible alterations of the skin at the site of contact. During the skin corrosion validation study of the EpiSkin test method, some *in vivo* corrosives were identified as non-corrosives *in vitro*. Since underclassification of chemicals may be due to non-specific reduction of MTT in solution, MTT interference corrections were performed on 5 chemicals detected as direct MTT reducers, indicating the need to adapt the EpiSkin skin corrosion test method by including specific controls for MTT reducers.

A stepwise testing strategy for the prediction of skin irritation and then skin corrosion was developed using the validated EpiSkin test methods to support the ongoing revision of the OECD test guidelines TG404 and TG431. When applying the testing strategy on about 50 reference substances (from the ECVAM validation studies), 20 *in vivo* irritant chemicals were identified *in vitro* as non-corrosive but correctly classified as irritants. In addition, 17 *in vivo* non irritants and non-corrosives were correctly predictive *in vitro* using both skin corrosion and irritation test methods. Finally 12 corrosive chemicals identified by NICEATM/ICCVAM as incorrectly predicted *in vitro* were evaluated. The results showed that corrosive chemicals misclassified in the *in vitro* corrosion test were identified *in vitro* as irritants.

This analysis of these new data should decrease the need for testing for both dermal skin corrosivity and skin irritation of substances for which sufficient evidence already exists.

I-11-137

Signature biomarker analysis for prediction of skin sensitizers using a cell-based *in vitro* alternative to animal experimentation

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Atopic contact dermatitis is a common inflammatory skin disease that affects a significant proportion of the population, and the incidence is increasing due to repeated exposure to sensitizing chemicals. The REACH regulation requires that all new and existing chemicals within the EU should be tested for hazardous effects. As the identification of potential sensitizers currently requires animal testing, this will have a huge impact on the number of animals needed for testing. Further, the 7th Amendment to the Cosmetics Directive (76/768/EEC) imposed a ban on using animals for testing cosmetic ingredients for all human health-related effects by 2013. Thus, development of reliable *in vitro* alternatives to animal experimentation for the assessment of the sensitizing capacity of chemicals is urgent.

We have developed a cell-based assay, based on the monocytic cell line MUTZ-3, for the purpose of testing the propensity of new chemicals to cause sensitization. We have stimulated the cell line with >40 skin sensitizers, irritants and controls for 24 h in optimal growing conditions (≥90% relative viability) and analyzed the activity with genome-wide transcriptional profiling. By employing advanced computational statistics, we have identified biomarker signatures which distinguish sensitizers from controls with 90% accuracy. Thus, we have identified a potent predictive biomarker signature for skin sensitization and demonstrated that the mRNA microarray is a powerful assay in itself. Being based on a human biological system, the assay is considered to be more relevant and more accurate for predicting sensitization in humans than the traditional animal-based tests. Further, the identified marker profiles are believed to describe biological pathways involved in sensitization.

I-11-142

Evaluating the sensitization potential of surfactants: Using *in vitro* methods in a weight of evidence approach

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An integral part of hazard and safety assessments is the estimation of a chemical's potential to cause skin sensitization. Non-animal test methods are in the process of being developed and formally validated. In order to gain more insight into the responses induced *in vitro*, representative surfactants were tested in methods that are in the prevalidation process. The battery of *in vitro* tests (animal data available) included peptide reactivity assays, the KeratinoSens assay, the hCLAT assay and EpiSkin irritation assays. Seven of eight surfactants were negative in the GPMT and in the KeratinoSens assay, seven of eight were positive in the LLNA, none formed covalent adducts with test peptides, and all were negative in the hCLAT assay. Six of eight surfactants would be rated as being irritants by the EpiSkin assay, seven of eight induced IL-1α, and all but one were positive in the LLNA based on ear swelling. A weight of evidence approach would classify seven of eight as being non-sensitizing skin irritants and would confirm that the LLNA tends to overestimate the sensitization potential of surfactants. As results obtained from LLNAs are considered as the gold standard for the development of new non-animal test methods, results such as these highlight the necessity to carefully evaluate the applicability domains of different test methods in order to develop reliable non-animal alternative testing strategies for sensitization testing. The results show how *in vitro* methods could possibly be used to interpret contradictory results from animal tests in a weight of evidence approach.

I-11-145

The Myeloid U937 Skin Sensitization Test (MUSST) for the prediction of skin sensitization potential

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Skin sensitization is a delayed type allergy consisting of a cellular immune reaction to small molecular weight chemicals, so far predicted using animal test methods such as the local lymph node assay (LLNA). In line with the 3Rs, *in vitro* alternatives are being developed based on early events of the skin sensitization process. One of these is the capacity of dendritic cells to recognize a chemical as a danger. The Myeloid U937 Skin Sensitization Test (MUSST) models this by measuring the up-regulation of CD86 expression on U937 cells. A chemical is classified as a sensitizer if it induces a dose-dependent up-regulation of CD86 expression at non-toxic doses in two concordant experiments. The MUSST prediction is exemplified with 2 sensitizers (phenyl benzoate, methylchloroisothiazolinone), 2 pre/prohaptens (ethylene diamine, eugenol) and 2 non-sensitizers (lactic acid, benzaldehyde) correctly classified by the assay. The predictive performances of the MUSST are evaluated with a panel of 50 reference chemicals (31 sensitizers and 19 non-sensitizers) against the LLNA data to support its submission to and its acceptance by ECVAM for a pre-validation. With the 40 classified chemicals (10 are inconclusive), the MUSST displays a concordance of 83% with 81% sensitivity and 84% specificity.

The performances, further evaluated with an extended set of 83 classified reference chemicals, show concordance, sensitivity and specificity above 75%.

The MUSST is an efficient assay for skin sensitization hazard characterization and is promising as a tool to be integrated within a battery of assays to perform a skin sensitization risk assessment.

I-11-152

Development of a skin sensitization test using a three-dimensional human skin model consisting of dendritic cells, keratinocytes and fibroblasts on collagen vitrigel membrane for application to cosmetic products

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Although several *in vitro* skin sensitization tests using human cells have been developed, it is difficult to use these to examine cosmetic products such as milky lotion and cream owing to their water insolubility. We have already established a test method using a three-dimensional human skin model consisting of normal fibroblasts, normal keratinocytes and normal dendritic cells on a collagen vitrigel membrane (VG-KDF-Skin-method). In this study, we compared this method with an *in vivo* method, and investigated the possibility of evaluating the skin sensitization potential of cosmetic products using this model.

The VG-KDF-Skin was treated with test chemical or cosmetic products for 1 h. After removal of these, this skin model was further incubated for 23 h. The supernatant was collected, and IL-1α, IL-4 and IL-8 were measured by ELISA. Test chemicals that showed over 150% of cytokine release compared to control were deemed to have a positive response.

Nine sensitizers and five non-sensitizers were examined. The accuracy, sensitivity and specificity of the VG-KDF-Skin method using IL-4 as an indicator vs. LLNA were 93%, 89% and 100%, respectively. The accuracy, sensitivity and specificity of the VG-KDF-Skin method, using IL-1α± vs. LLNA, were 50%, 56% and 40%, respectively.

Significant IL-4 release was induced, by model cosmetic samples containing a skin sensitizer of 2,4-dinitrochlorobenzene. The VG-KDF-Skin-method using IL-4 as an indicator would be useful for evaluating the skin sensitization potential of chemicals and products showing the properties of emulsions, creams or solids owing to their water insolubility.

I-11-153

Statistical prediction model for skin sensitization potential using an integrated dataset from h-CLAT, DPRA, and DEREK

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The validation of alternatives to animal testing for skin sensitization is ongoing and a number of testing methods are currently being developed. For such methods, the use of only a single alternative test method may not provide sufficient predictive performance to make an assessment of skin sensitization potential. As a result, it may be necessary to develop an assessment strategy which combines multiple forms of testing. The objective of this study was to confirm the predictive performance obtained using a parametric regression analysis of multiple testing results. A parametric regression model presents the advantage of easy interpretation, compared to non-parametric regression models such as neural networks or support vector machines. Multiple linear regression analysis and logistic regression analysis were performed via the Weka software using an integrated dataset for 101 chemicals obtained via LLNA, h-CLAT, DPRA, and DEREK. LLNA results (positive or negative) and LLNA EC3 values were selected as outcome variables. A Box-Cox transformation was applied to the variable data to improve both normality and predictive performance. Use of the logistic regression analysis for the prediction of LLNA results yielded predictive performance with an accuracy rate of 89.1%, a sensitivity rate of 93.4%, and a specificity rate of 76.6%. Use of the multiple linear regression analysis for the prediction of log-transformed LLNA EC3 values yielded a predictive performance with a multiple correlation coefficient of 0.791. By applying a statistical regression model to the dataset from LLNA, h-CLAT, DPRA, and DEREK, the predictive performance was improved over the prediction of the single test method.

I-11-169

Papain characterization: an approach to the cytotoxicity profile

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Papain is a vegetal enzyme applied in several medical devices due to its characteristic properties, such as proteolytic activity and cicatrization induction, which allow distinct biomedical applications. However, when dealing with compounds for human use, toxicity and biocompatibility studies are mandatory in order to assure efficacy and safety. On this account, in this work we report a study based on different commercially available enzymes. Each papain formulation was characterized in terms of biochemical properties, through enzymatic activity determination and protein content, as well as according to their physicochemical properties, through UV spectra and IR techniques, in order to identify possible differences in the enzyme, since distinct purification and stabilization methods are used at industrial level nowadays. The samples were subjected to a cytotoxicity assay with equivalent protein content and enzymatic activity. Human keratinocytes (HK) in high density were used to test papain at several concentrations (from 0.25 to 2% (w/v)) for 24 h of contact at 37°C, 97% humidity and 5% CO_2. The viable cells were measured by MTS/PMS and formazan product was quantified at 490 nm. The results revealed that despite the specific characteristics of each enzyme produced, regarding activity and IV and UV spectra, the cytotoxicity profile was similar for all samples. The enzymatic activity does not seem to play a major role in the cytotoxic effects, indicating that the characteristic cytotoxicity is related to the enzyme concentration. It is relevant to note that, under the evaluated conditions, all formulations were cytotoxic even at low doses.

I-11-173

Towards the 21st century: Advances and refinements in the prediction of sensitisation potential using the TIMES platform

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The TImes MEtabolism Simulator platform for predicting Skin Sensitisation (TIMES-SS) is a hybrid expert system that was developed at Bourgas University using funding and data from a consortium comprising experts from industry and regulatory agencies and coordinated by IQF. The model was developed with the aim of minimising animal testing, to be scientifically valid for regulatory purposes, and to be mechanistically transparent. TIMES-SS encodes 2D structure-toxicity and structure-skin metabolism relationships through a number of transformations, some of which are underpinned by mechanistic 3D QSARs. An external evaluation exercise was completed in 2007 where LLNA data were generated for 40 new chemicals and compared with predictions made by TIMES-SS. The results were promising with an initial concordance of 75%. An extensive evaluation followed to assess the results in light of reaction chemistry principles. The number of chemicals underpinning a given reaction chemistry alert was reviewed and four validation substances were subsequently tested. Recently, a 3-year research proposal was initiated as part of a new industry consortium. The skin (a) biotic metabolism simulators and the skin sensitisation model will be refined in light of new data and chemical insights. The applicability domain of the underlying experimental data will be evaluated to reflect current recognised inconsistencies between different *in vivo* assays. The feasibility of developing a respiratory sensitisation model will also be investigated. This presentation will provide an overview of the current status of TIMES-SS, highlight current refinement activities, and outline the strategy for deriving a respiratory sensitisation model based on preliminary investigations.

I-11-176

Evaluation of KeraSkin™-VM, a new reconstructed human epidermis model as an alternative to the skin irritation test method according to the OECD TG439

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Several alternative *in vitro* methods for the evaluation of skin irritants have been developed recently. The most promising one is the reconstituted human epidermal model. In July 2010, the OECD officially endorsed the validated reference method (VRM) using three 3D reconstructed human epidermis models, EpiSkin™ , EpiDerm™ SIT (EPI-200) and the SkinEthic™ RHE, as replacements for the *in vivo* skin irritation test. In this study, KeraSkin™ -VM model (MCTT Co, Korea), a new human epidermis model reconstructed using Asian skin tissues, was evaluated as another *in vitro* skin irritation test method using 20 reference chemicals according the performance standards of OECD TG 439. The test protocol was performed using the *in vitro* RHE-based test method with a minor modification, in accordance with the requirements of the performance standards of OECD TG439. The results obtained with the modified irritation protocol were comparable to those of VRM with the EpiDerm™ SIT (EPI-200) models. When comparing the performance of the KeraSkin™ -VM with UN GHS categories, an overall accuracy of 80%, sensitivity of 90% and specificity of 70% were obtained, and the accuracy of the KeraSkin™ -VM was comparable to that of the VRM. In this study, we demonstrated the new reconstituted human epidermal model KeraSkin™ -VM showed a good performance in terms of intralaboratory variability and predictive capacity to screen skin irritants. Further studies are on-going to improve the protocol and predictability for the interlaboratory validation study of KeraSkin™ -VM.

I-11-177

Development of a Fluorescence intensity Increased Method (FIM) to evaluate the skin photosensitization potential of chemicals

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In order to develop *in vitro* assays for the detection of the photosensitizing potential of chemicals, we tried to apply published *in vitro* sensitization assays to detect photosensitization. Among them, we focused on the ARE (antioxidant response element) assay and the SH test. The ARE assay is a luciferase-based assay using AREc32 cells which have an eightfold repeat of the ARE sequence as an upstream promoter of the luciferase gene. The SH test detects changes of cell-surface thiols by haptens using flow cytometry. Both assays were reported to be useful as *in vitro* sensitization assays.

Using the SH test, we evaluated changes of cell-surface thiols on THP-1 cells upon treatment with known photosensitizers and UVA exposure. Furthermore, we evaluated cell-surface amines in addition to cell-surface thiols. Using the ARE assay, we determined the optical condition of UVA exposure necessary to detect photosensitizers. As a result, we confirmed that most photosensitizers induced changes of cell-surface thiols or amines on THP-1 cells upon 5 J UVA exposure. However, piroxicam and p-aminobenzoic acid (PABA) did not induce changes of cell-surface thiols and amines under this condition but did induce ARE expression in AREc32 cells upon 2.5 J UVA exposure. The results of both assays using photosensitizers and non-photosensitizers were in good concordance with those of literature information.

In conclusion, our data suggests that changes of cell-surface thiols and amines on THP-1 cells and the ARE luciferase assay are useful to detect the photosensitizing potential of chemicals.

I-11-182

Evaluation of a photosensitizer by non-radioactive local lymph node assay (LLNA)

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Concerns over photoallergy of cosmetics and drugs are escalating, but validated alternative test methods for the detection of photosensitivity are yet to be developed. We investigated the modification of the non-radioisotopic local lymph node assay (LLNA) using BrdU with flow cytometry as a new alternative test method for the identification of photoallergic potential. Before a full-scaled experiment, proper test concentrations of test material were decided through a preliminary photo-irritation test. Treatment method and scheme were the same with LLNA. Mice received topical application of chloropromazine (CPZ, 0.01, 0.025, 0.1, 0.5 w/v%) or vehicle on both ears and were irradiated with ultraviolet A (UVA) afterward for 3 consecutive days. Each dosed group consisted of two sets – irradiated and unirradiated. Mice were sacrificed 24 h after intraperitoneal injection of bromodeoxyuridine (BrdU). Weight of ears and lymph nodes were measured to evaluate photo-irritation and lymph nodes were isolated and underwent lymphocyte preparation. Potential for photoallergy was determined by BrdU incorporation into lymph node cells, B/T cell ratio and *ex vivo* cytokine production. We regarded the test article as photosensitizer when SI was above 3 in the irradiated group and SI was below 3 in the unirradiated group. Stimulation index (SI) and cytokine release like IL-2 and IL-6 were significantly increased by CPZ at concentrations above 0.1%. In contrast, the B/T cell ratio was significantly increased from the lowest concentration (0.01%). Phototoxicity could be identified using ear swelling and cytokine profile changes. These findings suggest that photoallergic potential could be determined using SI, B/T cell ratio and cytokine production. Further studies with more diverse chemicals are on-going for the validation of the method.

I-11-186

An *in vitro* test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8

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Several studies have suggested that IL-8 is a biomarker to discriminate haptens from irritants. To develop a high throughput method to identify possible haptens, we established a stable THP-1-derived IL-8 reporter cell line, THP-G8, which translates SLO and SLR luciferase genes under the control of IL-8 and G3PDH promoters, respectively. After 6 h of treatment with chemicals, normalized SLO-LA (nSLO-LA) was calculated by dividing SLO luciferase activity (SLO-LA) by SLR-LA, and fold induction of nSLO-LA (FInSLO-LA) was calculated by dividing nSLO-LA with chemical treatment by that without treatment. THP-G8 increased nSLO-LA in response to LPS or several haptens. FInSLO-LA was positively correlated with IL-8 mRNA induction in THP-1 stimulated with LPS or haptens. When we examined the effects of 15 haptens and 7 irritants on nSLO-LA, however, THP-G8 significantly increased their nSLO-LA (FInSLO-LA≥1.4) by 13 haptens as well as 5 irritants. Interestingly, pretreatment of N-acetyl cysteine (NAC) suppressed increase in FInSLO-LA induced by all haptens (suppression index (SI) ≤0.8), while NAC did not suppress increase in FInSLO-LA by most irritants. Then we evaluated the performance of this reporter assay with the criteria of haptens as FInSLO-LA≥1.4 and SI≤0.8 more than 2 out of 3 independent experiments, which resulted in test accuracies of 82% for these 23 chemicals and 88% for the chemicals proposed by Casati et al. This newly developed assay would be a candidate to replace animal tests for skin sensitization because of its accuracy, convenience, and high throughput

I-11-187

CEFIC-LRI workshop on skin sensitisation methods

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Skin sensitisation is an important toxicological endpoint that is assessed for all chemicals. Due to animal welfare benefits and the ease of potency quantification, the LLNA (OECD 429) is commonly the first choice test for sensitisation testing. Moreover, under REACH, the use of other tests (i.e. the traditional guinea pig test (OECD 406)) needs to be scientifically justified. Discrepancies between results obtained with the LLNA and guinea pig tests have been reported for some classes of chemicals (e.g. surfactants, unsaturated fatty acids, siloxanes). Such substances are not considered sensitising based on historic test data and the absence of human evidence of sensitisation. These results suggest a need for improved characterization of test results to enable a better understanding of potential confounding chemistries. To broaden awareness among stakeholders, a CEFIC Long-range Research Initiative (LRI) workshop reviewed these experiences with a panel of experts from regulatory, academic and industrial organizations. Focused discussions involved the definition of "gold standard", applicability domains and the use of LLNA, guinea-pig and human experiences for the development of non-animal tests. The workshop findings and recommendations will serve as a guide in a research strategy to understand critical aspects of these test results and the development of reliable alternative methods, while advancing a flexible and intelligent skin sensitisation testing strategy across different chemical classes.

I-11-189

A strategy for the hazard identification and potency categorization of skin sensitization using a combination of non-animal tests

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The combination of several *in vitro* methods is a crucial approach in identifying the skin sensitization hazard adequately for a wide variety of chemicals without the use of animals. We have been developing an *in vitro* sensitization assay, the human Cell Line Activation Test (h-CLAT), emulating dendritic cell activation. In this study, we investigated a battery system: the combination of h-CLAT, the direct peptide reactivity assay (DPRA), an *in vitro* assay, and the *in silico* system, DEREK. Currently ECVAM is pre-validating both h-CLAT and DPRA. As a first step, the integrated testing strategy (ITS) was investigated. Final score calculated from the scores of each robust data set from each ITS component was used for the evaluation. ITS demonstrated a higher accuracy (85%) compared to DPRA, h-CLAT or DEREK alone. Secondly, the tiered approach using h-CLAT and DPRA were investigated as a practical system. The optimized tiered approach indicated the possibility of not only detecting the hazard but also for classifying the potency of chemicals. The predictivity for the potency classification was 72.3%, while the "under prediction" rate was especially low. Our results brought the non-animal testing system one step closer to replacing animal testing. Finally, we have been developing a novel *in vitro* test, EpiSensA, using a reconstructed epidermis model, which is expected to solve some of the current problems (i.e. lipophilic chemical evaluation). By adding EpiSensA to the tiered approach, the non-animal testing system will be used as an alternate to animal testing and will be leveraged in risk assessments.

I-11-190

ECVAM prevalidation study on skin sensitisation alternatives: progress update

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In the field of *in vitro* alternatives in toxicology, several alternative methods for acute local health effects have already been validated. In contrast, sensitization and other repeated dose endpoints have remained a significant challenge. However, recent progress with *in vitro* assays in skin sensitization toxicology has resulted in the development of mechanistically based test methods which could make a valuable contribution to the replacement of the existing animal tests. These approaches comprise the Direct Peptide Reactivity Assay (DPRA), the Myeloid U937 Skin Sensitization Test (MUSST) and the human Cell Line Activation Test (h-CLAT). Each of these test methods has been the subject of substantial evaluation including inter-laboratory assessments, and their status of development has led to their acceptance by ECVAM for inclusion in a Prevalidation Study in which the three test methods are challenged with a set of coded chemicals in three laboratories each. 24 chemicals, well characterised with respect to their sensitisation potential (or lack of), have been selected and will be tested once for the assessment of the between- laboratory reproducibility. A subset of 15 chemicals will be assessed a further two times for the evaluation of the within laboratory reproducibility. It is anticipated that results from the DPRA will be available late in 2011, whereas results from the cell-based assays are expected to be delivered during 2012. Assuming a successful outcome, future activity will require consideration of how to deploy these assays in a structured assessment of skin sensitization potential. An overview on the study organisation and progress will be provided.

I-11-194

Implementation of non-animal approaches for cosmetic safety assessments for skin sensitisation

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Skin sensitisation is a key endpoint for the safety assessment of cosmetic ingredients, and the mouse Local Lymph Node Assay (LLNA) is currently the standard test method that predicts the skin sensitising potential of chemicals as well as estimates of their relative potency. A key question that still remains to be addressed is to what extent skin sensitisation safety assessments can be made in the absence of animal data. Therefore, COLIPA, the European Cosmetics Association, undertakes efforts to continuously evaluate emerging tools and approaches in the field, comprising predictions based on physico-chemical properties (including *in silico* tools), indications for the presence or absence of structural alerts ((Q)SAR), read-across based on similar chemicals with available experimental data, *in vitro* methods, historical data and exposure-based waiving approaches (e.g. Threshold of Sensitisation Concern). These tools and approaches are integrated into safety assessment strategies such as the quantitative risk assessment (QRA) approach for skin sensitization, for which the analysis of the weight of evidence is considered as a basic element. Based on the outcome of a COLIPA workshop held in 2010, guiding principles are suggested in or-

der to further develop individual tools and how to combine them in order to finally enable safety decisions without the need for animal tests. The generation of data from alternative tools suited for hazard characterisation and potency evaluation is considered to be the key research area. The design of safety assessment strategies integrating all relevant information is complementing the extensive COLIPA research program that aims at developing and evaluating such alternative test methods.

I-11-198

The molecular mechanisms of IL-8 production by hapten-stimulated monocytes: analysis using a stable THP-1-derived IL-8 reporter cell line, THP-G8

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We have established a stable THP-1-derived IL-8 reporter cell line, THP-G8, in which SLO and SLR luciferase genes are regulated by IL-8 and G3PDH promoters, respectively. Using THP-G8, we developed a high throughput-screening test for haptens. To elucidate the mechanism of IL-8 induction by haptens, we stimulated THP-G8 cells with a representative allergen, 4-nitrobenzylbromide (4-NBB), and a non-hapten, sodium lauryl sulfate (SLS), together with various signal transduction inhibitors. MRS2578, the P2Y6 receptor-selective antagonist, and the ERK inhibitors PD980059 and U0126 significantly suppressed SLO-LA induction by both 4-NBB and SLS, consistent with recent reports of UDP involvement in the production of IL-8 via ERK activation. To understand the mechanism by which UDP is released from THP-G8 stimulated with chemicals, we used 9 chemical inhibitors against reactive oxygen species (ROS) (NAC and DPI), mitochondria complex I (rotenone), calcium signaling (BAPTA-AM), phosphoinositide-3-kinase (LY-294002), Rho-dependent kinase (Y-27632), and various Cl^- channels (glybenclamide, arachidonic acid, and GdCl3). All these inhibitors except for GdCl3 significantly suppressed 4-NBB-induced SLO-LA, while only NAC and DPI but not the others suppressed UDP-induced SLO-LA. The results suggest that UDP release by 4-NBB-stimulated THP-G8 is at least partly mediated by 1) ROS production by mitochondria, 2) intracellular Ca^{2+} elevation, 3) PI3K, 4) Rho-dependent kinase, and 5) some Cl- channels. Only DPI significantly suppressed SLS-induced SLO-LA, SLS releases UDP by a mechanism different from that of 4-NBB. These data suggest that THP-G8 could be a useful tool to investigate the molecular mechanism of IL-8 production as well as to identify skin sensitizers.

I-11-200

Study on development of *in vitro* photosensitization test using human-derived monocytes

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The European Parliament amended for the seventh time the Council Directive 76/768/EEC, which includes the ban of testing on animals and of the marketing of products/ingredients tested on animals. According to the framework of the 7th Amendment to this Directive, a timetable was laid out for the phasing-out of animal testing. This timetable states that the cut-off date for the marketing ban of cosmetic products tested on animals for UV-induced toxic effects including photosensitization is 2013. However, there is no regulatory alternative method for the photosensitization test. Thus, we performed this study to develop *in vitro* photosensitization using human-derived monocytes. Photosensitization is a delayed-type hypersensitivity reaction with an essential requirement for ultraviolet (UV) radiation. Some chemicals including some drugs are known to cause photoallergic reactions. In this study, we evaluated the expression of CD40 and CD54 in THP-1 cells exposed to known photoallergens, such as 6-methylcumarin and chloropromazine, to develop the new alternative method for identifying photosensitizing potential chemicals.

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I-11-202

In vitro skin sensitization test; human Cell Line Activation Test (h-CLAT)

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We have developed an *in vitro* skin sensitization test using THP-1 cells (human monocytic leukemia cell line), named "human Cell Line Activation Test" (h-CLAT). This test is based on the augmentation of CD86 and/or CD54 expression in THP-1 cells following exposure to sensitizers. We have evaluated about 100 chemicals, which have a different potential for skin sensitization, by h-CLAT and compared the results with LLNA or human test data. The accuracy of the h-CLAT vs. LLNA was over 84% and for h-CLAT vs. human test data was about 80%. Most chemicals were evaluated correctly, but a few chemicals were failures. The chemicals that were evaluated as "false-negative" were e.g. benzoyl peroxide, isoeugenol, phthalic anhydride, and abietic acid, and the chemicals evaluated as "false-positive" were 1-bromobutane and diethylphthalate. The result of "false-negative" might be caused by lacking metabolic activity in THP-1 cells, low solubility to water, and weak sensitizers. Next, we calculated the estimated concentration to induce marker expression with an RFI=150 for CD86 (EC150) or 200 for CD54 (EC200) in the h-CLAT, and these values were compared with the LLNA EC3 values. We especially classified into 2 groups, "strong" and "weak", on EC150=10. As a result, the strong group in h-CLAT has a high correlation with the extreme and strong groups in LLNA, and the weak group in h-CLAT has a correlation with the moderate and weak groups in LLNA. This result suggests that h-CLAT could be useful not only for hazard identification, but also for estimating chemical allergic strength.

I-11-206

Evaluation of SenCeeTox®, an integrative model for identifying chemical sensitizers

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Allergic contact dermatitis is the result of an adaptive immune response of the skin to direct exposure to an allergen. Because it is the most common manifestation of immunotoxicity in humans, a strict screening of all ingredients in consumer products is required. Current methods rely on animal testing (e.g. local lymph node assay) for determining chemical sensitization. Ethical concerns and regulatory changes in the EU have stimulated the development of alternative tests for the assessment of potential sensitizers. There is a common view that a strategy for the integration of the currently available methods will be required. Ceetox Inc. has developed an integrative approach, namely SenCeeTox®, allowing the EC3 value to be estimated and differentiating the degree of response from non-sensitizer (NS), weak (W), moderate (M), and strong (S), up to extreme (E). The purpose of this study was to evaluate the predictive capacity of this approach in a blinded manner. L'Oréal provided a set of 40 compounds (20 positive and 20 negative), consisting of 24 proprietary and 16 public domain chemicals that were assessed by CeeTox. All 40 compounds could be classified; those placed into E, S, and M were considered positive, while compounds classified as W or N were considered negative. Results obtained for the prediction of positive and negative compounds were promising. However, the model failed to accurately predict each sensitization category. Refinements and automation of the algorithm and the incorporation of additional assays should improve the model's ability to predict potency.

I-11-217

A combined model of sebocytes with human epidermal-dermal equivalents for evaluating the effect of topical application of sebum inhibitors

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Agents with sebum inhibitory activity could be used to prevent or treat acne and seborrhea. Once formulated, such agents can no longer be tested in monolayer cell cultures, and traditionally they were tested in animals. Thus, we developed an *in vitro* model system to evaluate topical, formulated agents for their effect on sebaceous lipids. This system utilizes epidermal-dermal equivalents which are overlaying cultured primary sebocytes. Following topical treatment, lipid production is quantified by Nile red, a fluorescent dye that selectively binds to neutral lipids. The predictability of the new *in vitro* system was validated using flutamide, a known agent with sebum inhibitory effect. A melanocortin receptor 1, 5 (MC1R, MC5R) antagonist, JNJ-10229570, was shown previously to inhibit sebaceous lipid production in ChT-induced primary sebocyte culture and in human skins-SCID mice. When the same JNJ-10229570 was applied topically for 5 days onto the epidermal-dermal equivalents overlaying the cultured sebocytes, a similar inhibitory effect was observed, verifying the usefulness of this new co-culture system. In conclusion, we developed an *in vitro* combination system for evaluating topical agents for their effects on the sebaceous lipids that replaces the use of animal models.

I-11-226

Methods development through recognition in 3Rs: L'Oréal commitment

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Dissemination of advances on alternative methods represents a step to promote alternatives to animal testing in line with the EU Cosmetics Directive. L'Oréal has, based on these principles, developed test methods to screen and test potential effects on chemicals.

We have focused initially on approaches for skin irritation. A peer review on various aspects of alternative techniques was performed at all stages of the R&D with a focus on *in vitro* methods improvement of chemicals selection (screening) as well as quality testing. To ensure quality and objectivity, experts from international committees oversee the content of EpiSkin and SkinEthic RHE protocols. Details of the approach will be presented for both skin corrosion and irritation with a set of 50 reference chemicals. Using computational approaches, an automated workflow algorithm was developed to predict a molecule's potential for skin irritancy based on *in vivo* Draize data. The practical approaches developed by L'Oréal in the areas of eye irritation (SkinEthic HCE defined with 90 chemicals), skin sensitization (MUSST assay optimized with 50 chemicals), phototoxicity and genotoxicity will be described. For chronic and systemic toxicity testing, a realistic approach relied on the combination of data generated for multiple endpoints. Preliminary studies indicated that the method had good sensitivity and specificity (91% and 78%) while defining a LD_{50} threshold at 2000 mg/kg.

Combination of *in silico*, read across and *in vitro* strategies assure realistic scientific approaches suitable for the safety assessment process within industry.

I-11-230

Contact sensitizers modulate the arachidonic acid metabolism of PMA-differentiated U-937 monocytic cells activated by LPS

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Contact sensitizers are defined as reactive molecules that have the ability to modify skin proteins to form an antigen. In addition to the haptenation mechanism, inflammatory signals, leading to the activation of dendritic cells, are described to be crucial for the effective induction of an antigen-specific T cell immune response. However, the sensitization phase is often a silent process, without obvious clinical manifestations of inflammation/irritation. Even more, anti-inflammatory properties of some molecules do not prevent them from inducing skin sensitization. The aim of this study was to better understand how sensitizers modulate an inflammatory response. To address this purpose, we used the human monocytic-like U-937 cell line differentiated by phorbol myristate acetate (PMA) and investigated the effect of 6 contact sensitizers (DNCB, PPD, hydroquinone, propyl gallate, cinnamaldehyde and eugenol) and 3 non-sensitizers (lactic acid, glycerol and tween 20) on the production of pro-inflammatory cytokines (IL-1β and TNF-α) and on the arachidonic acid metabolic profile after bacterial lipopolysaccharide (LPS) stimulation. Our results showed that among the tested molecules, all sensitizers specifically prevent the production of PMA/LPS-induced COX-2 metabolites (PGE_2, TxB_2 and PGD_2). We further demonstrated that there is no unique PGE_2 inhibition mechanism: while the release of arachidonic acid (AA) from membrane phospholipids does not appear to be a target of modulation, COX-2 expression and/or COX-2 enzymatic activity are the major steps of prostaglandin synthesis that are inhibited by sensitizers. Altogether these results add a new insight into the multiple biochemical effects described for sensitizers.

I-11-234

Computational system for predicting chemical reactivity towards macromolecules and subsequent adverse effects

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The ultimate goal of this work is the development of a computational tool for predicting chemical reactivity towards macromolecules (proteins, DNA) and subsequent adverse effects such as skin sensitization, genotoxicity, etc. Firstly, we focused on contact dermatitis, which results from the interaction between a hapten (electrophile) and the side chain of nucleophilic amino acids of proteins. Therefore the reactivity of chemicals towards peptides was studied. The domains of reactivity categories are defined according to the types of interaction mechanisms such as Michael addition, epoxide ring opening, Schiff-base formation, acylation, etc. The chemicals acting by these mechanisms have specific structural functionalities with different hardness/softness, which can be assessed by quantum-chemical parameters and used to predict chemical reactivity towards macromolecules. Experimental data of reactivity of chemicals on synthetic cysteine/lysine peptides (Direct Peptide Reactivity Assay) and/or glutathione are used to calibrate the boundaries of defined reactivity categories as well as for defining new categories for protein binding potency. The ability of chemicals to interact by ionic or radical mechanisms were assessed by quantum-chemical parameters evaluating the stability of formed intermediates and hence, the energetic feasibility of the respective transformations. The classification of parent chemicals according to the reactivity categories will be used to provide a hazard assessment for subsequent adverse effects.

I-11-243

Use of melanocytes and keratinocytes in co-culture for the assessment of the potential of a Brazilian flora nut as a de-pigmentation agent

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Popular reports indicate the use of a nut from Brazilian flora as a skin depigmentation agent. This study proposes to use human melanocytes and keratinocytes in co-culture *in vitro* and to treat them with extracts obtained from the nut's shell. L-DOPA, a stimulator agent of melanogenesis, the melanin production pathway responsible for skin pigmentation, was standardized in co-culture of melanocytes and keratinocytes as standard control for the presence of melanocytes by pigmentation. To obtain the extraction samples the nut shells were crushed and heated in an incubator at 200°C to give two viscous extracts with different major components, called extracts (A) and (B) respectively. Based on topical use cream formulations developed in previous studies, tests of solubility of the extracts are being carried out, using the same concentrations of the formulations in the culture medium. Murine fibroblasts, human melanocytes and keratinocytes are being tested in co-culture and then treated with extracts (A), (B), negative and positive control, in order to assess the depigmentation property of the extracts as inhibitor of melanogenesis by DOPA reaction test. Hydroquinone, a known inhibitor of the melanogenic pathway, has been used as positive control of extracts (A) and (B), which subsequently will have their results compared to hydroquinone.

I-11-245

Use of fresh, functional human skin tissue in assessing anti-inflammatory effects of human pharmaceuticals

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It is estimated that inflammatory disorders involving the skin account for 15% of all visits to general practitioners in Europe. Experimental research in human skin disorders is generally performed using animal tissue or a synthetic human skin preparation. While advancements in treatments have been made using such models, these are not without their disadvantages. Herein, we present cytokine data obtained from fresh, full thickness human skin biopsies which were exposed to a UV light source or an inflammatory chemical.

Fresh human skin from cosmetic procedures was obtained with full consent. Full-thickness skin biopsies were placed into a transwell filter in a 24-well culture plate with 1 ml of culture medium with the epidermis facing upwards and the dermis suspended in the culture medium. Biopsies were either exposed to a UV light source (253 nm) or had lipopolysaccharide (LPS; 1, 10 & 100 μg/ml) or phytohemagglutinin (PHA; 10, 100 & 1000 μg/ml) applied to the culture media to artificially inflame the skin. Biopsies were cultured for up to 24 h at 37°C in 5% CO_2/air with the supernatant samples obtained at various time points and cytokine levels assessed by ELISA. Exposure of skin to UV light for 15 min or the addition of LPS or PHA to the culture media was generally shown to cause an increase in all cytokines and immunomodulators studied, with PHA shown to have the most marked effects. The results presented above demonstrate that full-thickness human skin can be used to model inflammatory skin conditions.

I-11-259

Peptide reactivity assay using spectrophotometric analysis for screening of skin sensitizers

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Evaluation of the skin sensitization potential is an important step for the safety assessment of new ingredients in cosmetics and topical drugs.

Haptenation is a determinant step in the induction of skin sensitization. Thus, tests to measure reactivity of chemicals with peptides or proteins using HPLC and or LC/MS have been developed as an *in vitro* skin sensitization testing method. In this study, we tried to examine the possibility of spectrophotometric analysis for evaluating the peptide reactivity using two kinds of synthetic peptides, Ac-RFAACAA and Ac-RFAAKAA, with 30 chemicals. Free thiol and amino groups of the non-reacted peptides were measured by UV-VIS spectrophotometer using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and by fluorometer using fluorescamine™ after reaction with chemicals.

Most sensitizers reacted with cysteine peptide and some chemicals reacted with both peptides or with only lysine peptide. Glutaric dialdehyde and benzocaine highly depleted lysine peptide but did not have much effect on cysteine peptide. Most of the non-sensitizers showed a low depletion rate for both peptides. Therefore, results from two model peptides should be integrated for the understanding of sensitization potential. These results suggested that the peptide reactivity test using spectrometric methods could be an easy, fast and high throughput screening tool for the prediction of skin sensitization potential.

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I-11-274

Human hair follicle equivalents *in vitro* for substance testing

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The human hair follicle plays an important role in skin biology. Being highly vascularized and surrounded by dendritic cells, it supports penetration of substances into the skin and further into the bloodstream. The continuous implementation of test procedures on human skin equivalents into OECD guidelines for substance testing was the first move toward animal free testing of cosmetics/chemicals.

Substance testing on human hair follicles *in vitro* can add significant value to the current test procedures. By carefully analyzing and recapitulating the growth and differentiation mechanisms of hair follicle formation, we recreated human hair follicles in tissue culture that were capable of producing hair shaft and revealed a striking similarity to their *in vivo* counterparts. Extensive molecular and electron microscopy analysis were used to track assembly of follicular keratinocytes, melanocytes and fibroblasts into the final hair shaft, producing microfollicle architecture. The hair follicle generation process was optimized in terms of efficiency, reproducibility and compliance with regulatory requirements for later transplantation. In addition, we developed a procedure to integrate the *de novo* created human micro-follicles into our existing human skin equivalents for substance testing. Tissue culture data, histo- and immunostaining of the organotypic cultures as well as marker analysis are presented. The later use of the established system for the evaluation of the role of hair follicles in dermal substance transport mechanisms for cosmetic and pharmaceutical products will be discussed.

I-11-276

A new perspective to evaluate sensitizing agents using microarray analyses

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The need for standard methods to assess undesired effects and the increasing public interest to avoid using animals for substance testing has led researchers to turn to cell-based methods. Cells obtained directly from donors are usually limited in quantity, therefore permanent cell lines have been tested as alternative assays. In this study, the U-937 permanent human monocytic cell line was used to analyze the gene expression response to four compounds showing different sensitizing activity in parallel to a test botanical extract. Global gene expression response was assessed using DNA micro-arrays. Salicylic acid and eugenol produced relatively weak responses, which could not be differentiated from the control treatment, whereas in the citronellal treatment, from 100 genes differentially expressed (fold change, FC > ±2) 90 genes were upregulated, including 37 genes involved in inflammatory response. In the propyl gallate and botanical extract treatments, 2955 and 414 genes, respectively, were identified as differentially expressed (FC > ±2) and are related to cell cycle and general cell maintenance. 87% of the genes modulated by the botanical extract were common to propyl gallate. 40 genes are modulated by both citronellal and propyl gallate. However, 35 of them show inverse regulation, i.e. the genes upregulated by citronellal are downregulated by propyl gallate and *vice versa*, indicating that these substances sensitize cells by different mechanisms. These results indicate that cells can show different gene expression responses to sensitizing substances and any set of diagnostic genes needs to consider genes involved in various pathways and biological functions.

I-11-280

Evaluation of SENS-IS®, an Episkin® based model for identifying chemical sensitizers

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In the context of the 2013 ban given by the EU Cosmetics Directive, the ability to identify and classify the skin sensitization potential of chemicals without animals is of high importance for the cosmetic industry. A range of different *in vitro* chemistry-based (DPRA, GSH reactivity) and cell-based methods (MUSST, hCLAT, Keratinosens) have been developed and we are currently evaluating some of them for their applicability to cosmetic ingredients and physicochemical diversity. Although these assays appear to be promising for hazard identification, potency assessment is still limited. Possible limitations may be linked to the metabolism that may differ between the models and native skin, to bioavailability, which is not considered in monolayer cultures, and to the danger signal that may be different in monolayers as compared to a natural tridimensional microenvironment.

ImmunoSearch developed SENS-IS, a new method based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermis (Episkin®), thus providing a possible way to encompass these limitations and come closer to potency assessment. With the aim of evaluating the predictive capacity of this approach on a cosmetic ingredient constituted set, L'Oréal provided in a blinded manner a set of 40 proprietary as well as public domain chemicals that were assessed by ImmunoSearch. We present here the results of this study and will analyze the genomic signatures among chemical and ingredient classes.

I-11-281

Enterprise and university join efforts to develop *in vitro* alternative methods in Brazil

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Modifications in the regulation for cosmetics in Europe prohibiting animal tests for evaluating the sensitization potential of chemicals and new ethical standards of society compelled us to start developing *in vitro* alternative methods. In Brazil, regulatory boards, universities and society are also discussing alternative methods and their relevance for product development. To accelerate the Brazilian participation in that field of research, Natura Innovation and Products Technology Ltd and the Federal University of São Paulo joined efforts and established a partnership to study an *in vitro* model to replace an animal test. Our aim is to distinguish a sensitizer from a non-sensitizer substance using dendritic cell lines. In this way, 400 nM phorbol myristate acetate (PMA) was used to differentiate the human monocytic leukemia cell line (THP-1) and the human histiocytic lymphoma cell line (U937). After checking the pattern changes in the marker expression on THP-1 and U937 cells, such as CD14 and CD1a, differentiated cells were incubated with two strong sensitizers (dinitrochlorobenzene, p-phenylenediamine), three moderate sensitizers (methyl-chloro-isothiazolinone, methyl-isothiazolinone, cinnamaldehyde), two weak sensitizers (citronelal, citral) and two non-allergens (SDS, lactic acid). For each chemical, 5 concentrations were used in order to give a predicted cell viability range of 20-95%. Preliminary results showed that the expression pattern of CD86/CD54, as well as that of the secretion of IL-1β, IL-8, IL-18 differed depending on the chemical.

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I-11-293

Development of SENS-IS®, an Episkin® based model for measuring chemical sensitizer potency

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For the cosmetic industry, due to the ban starting in 2013 given by EU Cosmetics Directive, the ability to identify and classify the skin sensitization potential of chemicals without animals is of high importance. A number of assays has been developed and are currently under evaluation. However their ability to assess sensitization potency is limited. These limitations might be due to the use of monolayer culture and not native human skin. To overcome these limitations we developed SENS-IS, a new method based on the quantitative analysis of specific biomarkers expressed in 3D reconstructed epidermis (Episkin®). The selection of biomarkers was done by analysis expression profiles of mouse ear skin treated with several sensitizers and irritants. These studies were completed by analysis of mRNA expression in suction blisters sampled from sensitized patients challenged with $NiSO_4$ or fragrance mix or SLS. The first selected panel of biomarkers was then further refined on monocytic cell lines challenged with chemical or on 3D reconstructed epidermitis. We will present here the results of these studies and will show the predictive capacity of this approach on a set of 50 chemicals selected from a panel of perfume ingredients.

I-11-301

Characterization of alcohol- and aldehyde-deshydrogenase activities in normal human skin compared with reconstructed human skin models

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Skin is considered the body's envelope and a physical barrier to its environment. However, it contains numerous metabolizing enzymes which give it a potential role in terms of metabolism and detoxification. The 7th amendment to the European Cosmetic Directive bans the use of animal testing to evaluate the efficacy and safety of new cosmetic ingredients. This policy has forced the cosmetic industry to develop reconstructed human skin models (skin models) as tools for alternative methods to animal experimentation. For this reason, the models need to be characterized and compared with normal human skin (NHS) in terms of metabolic capabilities. In this work, we characterized alcohol deshydrogenase (ADH) and aldehyde deshydrogenase (ALDH) activities. Previous studies showed that NHS and reconstructed human epidermis such as Episkin™, SkinEthic-RHE™ and the full thickness model of Episkin™ expressed several ADH and ALDH isoforms. Their global catalytic activities were quantified in NHS and skin models using cinnamyl alcohol and cinnamic aldehyde as substrates, respectively. Apparent V_{max}, K_m and ratio V_{max}/K_m (estimating metabolic clearances) were calculated for each tissue from metabolite measurements of dose effect studies. Results showed that in NHS and in skin models, ADH and ALDH enzymes are functional and that ALDH activity is more important than ADH activity (ratio V_{max}/K_m ALDH > ratio V_{max}/K_m ADH). To conclude, the skin models can be easily used to study the detoxification process of primary alcohols or aldehydes, considered as potential sensitizers, and define their levels of cytotoxicity at the skin level.

I-11-304

In vitro alternative for chemical allergenicity screening using plasmacytoid dendritic cells

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Human dendritic cells (DC) have been used as an alternative to existing animal models for contact sensitization. Such methods are necessary to comply with the ban on animal testing imposed by the Cosmetics Directive in the EU. We investigated whether normal human plasmacytoid DC (pDC) can be used to identify contact allergens. pDC were exposed to chemical allergens (n=49) or irritants (n=42) and the highest concentration that yielded >50% viability was used in the study. Allergens were identified based on the stimulation index (SI) calculated by the fold increase in CD86 expression levels. A material that had an SI ≥1.5 in at least 50% of the pDC donors (n=2-5 donors) was considered an allergen. Historical LLNA and human clinical data were available for 75 of the 91 materials. An SI of ≥1.5 fold was obtained for 41 of 43 allergens but not for 26 of 32 non-allergens. Based on the results, a prediction model was developed for chemical allergenicity. The pDC assay has sensitivity = 95%, specificity = 81%, and accuracy = 89%; these results were comparable to the standard LLNA assay. Transferability of the test method was evaluated using 7 test articles in 3 laboratories. The results showed all samples were correctly identified. In conclusion, the CD86 expression level in pDC appears to be a sensitive and specific predictor of allergenicity. These results will be submitted to ECVAM for inclusion in a formal validation study.

I-11-313

In vitro testing of contact allergens: Which cell types are suitable?

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Allergic contact dermatitis (ACD) is an adaptive inflammatory response of the skin triggered upon exposure to certain chemicals or metal ions. Since many ingredients in consumer products might exert allergenic potency, there is a need for appropriate screening and characterization of these chemicals. Still, up to now the identification of potential allergens completely relies on animal testing. Due to economical and ethical reasons, however, the development of *in vitro* test systems for identification of potential sensitizers is mandatory.

Since dendritic cells (DCs) play a pivotal role in the initiation of ACD, several attempts were made to use monocyte-derived DCs and, due to easier handling, cell lines with DC-like properties. However, only few investigations exist that focused on the suitability of different cell types for their use in *in vitro* test systems. In the present study we compared monocyte-derived DCs and monocyte-derived Langerhans cells with the widely applied cell lines Mutz-3 and THP-1, respectively.

Four known allergens were tested for their ability to alter the expression of several immunomodulating surface molecules. We used multicolor flow cytometry to detect differences in expression patterns of surface markers that were previously associated with cell maturation. In addition to the upregulation of CD86, we observed both a dose-dependent upregulation of programmed death ligand 1 (PD-L1) and a downregulation of the dendritic cell immunoreceptor (DCIR). While both monocyte-derived cell types displayed highly significant changes in the expression levels of these surface markers upon exposure to allergens, the corresponding changes observed in cell lines were much smaller.

I-11-329

Skin-sensitizing capacity and potency: pre-validation of an alternative two-tiered *in vitro* assay

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Due to new legislation, the use of *in vivo* models to assess the allergic potential of chemicals is under debate. Animal models are not capable of distinguishing skin sensitizers from non-skin sensitizers. Earlier studies show that production of IL-18 by NCTC2544 keratinocytes could provide an *in vitro* tool to discriminate between contact and respiratory allergens and irritants (Corsini et al., 2009). Spiekstra et al. (2009) developed an Epidermal Equivalent (EE) model that is capable of determining the irritating potency of a chemical by measuring the production of IL-1α. Since IL-18 cannot be a marker for irritating potency and IL-1α cannot be used to distinguish between skin sensitizers and non-skin sensitizers, a two-tiered approach was developed to predict whether a chemical is a potential skin sensitizer and how strong this chemical reacts.

Pre-validation of Tier 1: During the inter-laboratory transfer phase of the pre-validation, a total of 5 individual laboratories tested 4 different chemicals to distinguish the skin sensitizers from non-skin sensitizers. All 5 labs were able to recognize the non-skin sensitizer. False negative results showed that the readout of fold increase in IL-18 production, compared to the vehicle, might not be sufficient to recognize skin sensitizers. Therefore, using dose-response data might be a better option to use as readout.

Tier 2: Currently, 5 laboratories are working on the inter-laboratory transfer of the EE model. Two different skin sensitizers will be tested, i.e. 1 extreme and 1 moderate skin sensitizer, to determine whether the model can rank skin sensitizers according to their potency.

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I-11-332

VITOSENS: a mechanism-based *in vitro* assay for chemical-induced skin sensitization

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VITOSENS is an *in vitro* assay based on exposure-induced expression changes of gene transcripts in dendritic cells derived from $CD34^+$ progenitor cells in human cord blood. The assay was initially designed as a classifier and it is able to discriminate chemical skin sensitizers from non-sensitizers (Hooyberghs et al., 2008). Moreover, by combining different VITOSENS variables we were able to model an *in vitro* potency value that closely fits *in vivo*-derived data, and over the entire range from weak to extremely sensitizing chemicals (Lambrechts et al., 2010a). As such, the assay can provide valuable information in the context of chemical risk assessment. Finally, a reliable test system should be based on key elements of the *in vivo* disease process it is screening for. We demonstrated the functional relevance of the *in vitro* VITOSENS gene markers. In a first step we proved their differential protein expression (Lambrechts et al., 2010b) and in a second phase we evaluated changes in DC maturation after pharmacologically counteracting the sensitizer-induced activity of the markers (Lambrechts et al., in press). In conclusion, these results point to the feasibility of applying VITOSENS as a mechanism-based *in vitro* assay to classify chemicals according to their inherent sensitizing risk. The assay can represent the antigen-presenting aspect in an integrated approach towards an alternative for skin sensitization testing.

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I-11-334

Development of PEPT-IS®, a peptide-binding based assay for assessing chemical sensitization using lipocalin derived peptides

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For the cosmetic industry, due to the ban staring in 2013 imposed by EU Cosmetic Directive, the ability to identify and classify the skin sensitization potential of chemicals without animals is of high importance. A key step in the skin sensitization process is the formation of a covalent adduct between the skin sensitizer and endogenous proteins and/or peptides in the skin. A number of assays have been developed and are currently under evaluation, but their ability to predict weak sensitizers is poor. To augment sensitivity of the assay we selected a peptide from skin-expressed lipocalin proteins. The lipocalin protein family is a large group of small extracellular proteins with the ability to bind a range of small hydrophobic molecules that bind to specific cell-surface receptors and form complexes with soluble macromolecules. In the context of sensitization, lipocalins display a number of interesting characteristics. Members of the lipocalin family, odorant binding protein, bind a number of sensitizers, numerous animal-derived allergens are lipocalins and, finally, we observed that NGAL or lipocalin 2 is specifically expressed in skin challenged with sensitizers. We selected a peptide from a conserved region that contains homologies with the epitopes recognized in animal allergens and that contains cystein and lysine amino acids. Using this peptide we developed PEPT-IS®, a peptide binding assay using a single peptide with 4 h incubation. We will present here the results of these studies and will show the predictive capacity of this approach on a set of 30 chemicals.

I-11-338

Gene profiles of a bronchial epithelial cell line (BEAS-2B) induced by exposure to low-molecular weight chemicals

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Being the first cells that encounter xenobiotics that enter the body through inhalation, the major function of the airway epithelium was once thought to be that of a physical barrier. However, the epithelium also responds actively to changes in the external environment, e.g. the presence of low-molecular weight (LMW) chemicals, by secreting a large number of molecules and mediators that signal to cells of the immune system. To obtain more insight into the role of the respiratory epithelium on a molecular level, gene profiles of a bronchial epithelial cell line (BEAS-2B) were identified after exposure to a panel of LMW chemicals. BEAS-2B cells were exposed during 6, 10, and 24 h to a panel of 18 LMW chemicals (i.e. 9 respiratory sensitizers, 4 irritants, and 5 skin sensitizers). Overall changes in gene expression were evaluated using Agilent Whole Human Genome 4x44K oligonucleotide microarrays. Analysis of the identified gene profiles were performed by means of GOFFA (gene ontology for functional analysis) and pathway analysis tools. Furthermore, Fisher Linear Discriminant Analysis (LDA) was used to reveal gene signatures that can discriminate between different chemical classes related to challenge specific properties.

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I-11-386

Integrated adaptive testing strategy for skin sensitization assessment

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Bayesian Network Integrated Testing Strategy (ITS-1) pilot phase showed potential to be a useful tool for decision making with alternative test information and offered novel insights. In a next generation ITS, ITS-2, the aim was to confirm findings from the pilot phase using a significantly enlarged data set and to develop a practical, optimal testing strategy for skin sensitization hazard testing. To this end, we continue to combine *in silico*, *in chemico* and *in vitro* data related to skin penetration, peptide reactivity, activation of Nrf2-dependent gene activity and dendritic cell activation. However, we have introduced several changes. We have extended the number/modified input tests as they have evolved over time. For example, we evaluated the value of the newly developed peroxidase peptide reactivity assays that consider metabolic activation and report the results based on dose-response with one of the existing direct peptide reactivity assays. We have replaced the AREc32 assay with the more standardized KeratinoSens assay. In addition, we have included cytotoxicity as a cofactor that may be an indicator of effects related to danger signal formation / local trauma thought to affect sensitizer potency. We also include mechanistic evidence, such as reactivity domain characterization, which is not directly related to potency yet as an important co-factor in potency determination. In this way we have started the evolution from an integrated testing strategy towards an intelligent testing strategy that will rely on increasing mechanistic evidence generated with systems biology data.

I-11-397

Predicting sensitizing potential of cosmetic ingredients: enlargement of the applicability domain of the MUSST assay by using complementary U937-based assays

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Allergic contact dermatitis resulting from industrial, environmental or domestic exposure to sensitizers is the most common manifestation of immunotoxicity in humans. Skin sensitization risk assessment and, more precisely, the sensitizing potential of ingredients used in the cosmetic and pharmaceutical industries so far essentially relies on available animal test methods, such as the mouse local lymph node assay. In the context of the 7th amendment to the Cosmetic Directive as well as the recent EU-legislation on chemicals (REACH), the cosmetic industry is particularly concerned by the challenge of finding *in vitro* alternatives to assess the sensitizing potential of chemicals.

Contact sensitizers induce several phenotypic and functional changes on dendritic cells (DC) *in vivo* and *in vitro*. One of these changes, the induction of CD86, is the most frequently analyzed endpoint for the *in vitro* prediction of contact sensitizers using different cellular models based on DC or human myeloid cell lines. We developed the Myeloid U937 Skin Sensitization Test (MUSST) based on the induction of CD86 on U937 cells. Years of in-house experience with this assay led us to identify its limits, and to develop further methods and further models (including 3D-models) to overcome these limits. We will describe here how an adequate use of CD86-mRNA test, U937-apoptosis assay and the Episkin-U937 co-culture assay can complement the MUSST assay to enlarge its applicability domain and thus to cover a larger physicochemical diversity encountered in cosmetic ingredients.

I-11-439

In vitro skin irritation testing of greasy and sticky substances

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Skin irritation evaluation is an important consideration for safety assessment and is therefore required by various regulatory authorities for notification and import of test substances. The objective of the present study is to improve the skin irritation test validated by ECVAM for plant extract testing.

We suggest that the washing step must be revised since the plant extracts are more lipophilic and that a positive irritancy result could be due to a longer time of exposition than to an irritancy potential itself. For that purpose, different ways of washing were tested in addition to controls other than those validated by ECVAM. Reconstructed human epidermis (RHE) Skinethic® samples were used, and were topically exposed to substances for 42 min. Then the RHE were washed normally (removing the excess of substance with a cotton-tip and 25 ml PBS) or specially (removing the excess of substance with a cotton-tip soaked with mineral oil, DMSO, or SDS 0.1% solution, then with 25 ml PBS). The parameters evaluated were viability (MTT test), IL-1α secretion (ELISA) and histology.

Two lipophilic controls were selected: Vaseline, as negative control and N Alkyl "suif" as positive control. RHE exposed to Vaseline have a weaker cell viability (-20%) than those exposed to PBS, highlighting that lipophilic substances could have a higher cytotoxic potential than hydrophilic substances. We tested different washes on these controls and concluded that the best washing procedure was with SDS 0.1%: it permits the effective removal of the excess of product from the surface without altering viability.

I-11-446

Updated NICEATM evaluation and international acceptance of the reduced murine local lymph node assay

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To minimize allergic contact dermatitis (ACD) occurrence, regulatory authorities require testing to identify substances with ACD potential. Such substances must be labeled with the hazard description and precautions necessary to minimize exposure. The murine local lymph node assay (LLNA) is an alternative test method for determining the ACD hazard potential of most types of substances and, compared to guinea pig tests, requires fewer animals, less time, and eliminates pain and distress. The reduced LLNA (rLLNA), which uses only the high dose, reduces animal use by a further 40% compared to the multidose LLNA. LLNA results from 1071 published and unpublished studies, representing 664 unique substances, were obtained. Accuracy for the rLLNA was 98.4% (1054/1071), with false positive and false negative rates of 0% (0/319) and 2.3% (17/752), respectively. These results reinforce ICCVAM's 2009 recommendation (which was based on 471 LLNA studies) that the rLLNA be routinely considered before conducting the multidose LLNA, when dose-response information is not required. Based on the ICCVAM/NICEATM joint evaluation, the rLLNA was included in an updated version of the OECD Test Guideline for the LLNA (TG 429) that was adopted in 2010. The availability of this international TG will allow for global use of the rLLNA for regulatory testing, which is expected to significantly reduce animal use for ACD hazard testing while supporting the protection of human health.

The views above may not represent the official position of any government agency. ILS staff supported by NIEHS contract N01-ES-35504.

I-11-456

Effect of skin barrier function and metabolic ability on the concentration-distance profiles of chemical compounds in reconstituted cultured human skin models

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Reconstituted cultured human skin models (RMs) have been used for *in vitro* skin corrosion/irritation tests. However, false-negative and false-positive reactions were obtained for the tests with several chemical compounds. We have already reported that the skin viability (skin irritation) showed a fairly good relationship with skin concentration of chemical compounds. In the present study, therefore, we examined reasons for the false-positive or -negative reactions under the assumption that the main reasons would be differences in concentration-distance profiles of the compounds between RMs and human skin.

An *in vitro* skin permeation experiment was performed to obtain permeability coefficients through whole skin (P_s) and stratum corneum-removed skin (P_{ved}). Several hydrophilic compounds and ethyl nicotinate as a model ester compound were used. Obtained esterase activity parameters (K_m, V_{max}) were compared between RMs and human skin.

The permeability coefficient ratios (P_{ved}/P_s) of hydrophilic compounds in RMs were much lower than those in human skin, although Ps value in RMs was almost the same in human skin. This result suggests that viable epidermis has high barrier function, as in the stratum corneum, for the RM permeation of hydrophilic compounds, which is a reason for the false-positive reaction. Esterase activity (V_{max}/K_m) in RMs was much lower than in human skin. False-negative results in RMs may be obtained when the parent compounds have skin irritation properties. Thus, differences of concentration-distance profiles of compounds between RMs and human skin must be considered to explain false-positive and -negative reactions in skin irritation tests.

I-11-458

Establishment and characterization of *in vitro* skin models mimicking hallmarks of atopic skin

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Atopic dermatitis (AD) belongs to the major health problems in the industrialized world with currently 10-20% of the children and 1-3% of the adults being affected. Loss-of-function mutations in the filaggrin gene (FLG) are a strong predisposing factor for AD, although its relevance for the pathomechanism is not yet understood in full. The generation of an *in vitro* model which exhibits hallmarks of atopic skin would allow for further evaluation of underlying pathogenetic mechanisms, for testing of new treatment options, and for toxicological studies in a simple, fast and cheap way.

In this study we have knocked down FLG expression in normal, human keratinocytes and investigated its impact on epidermal maturation and on the response to skin irritation in 3D skin models.

Histopathological evaluation showed disturbed epidermal differentiation and maturation in the knock down model. In contrast, in healthy tissues all relevant dermal structures were developed nicely. Moreover, skin irritation induced by an application of sodium dodecyl sulphate resulted in significantly higher LDH-leakage and IL-6/-8 levels ($p<0.001$) in the knock down models. This is well in accordance with the *in vivo* situation where the skin of an atopic patient shows higher susceptibility to skin irritation compared to non-atopic individuals. This study clearly demonstrates that deficiencies in FLG expression considerably impair skin barrier development and trigger skin irritation based on inflammatory responses. This FLG knock down construct is a first step towards the development of atopic-like skin *in vitro* model.

I-11-535

Retrospective analysis of the EpiDerm 3-minute prediction model for assessment of GHS skin corrosion packing group sub-category 1A

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OECD has adopted several ECVAM-validated reconstructed human skin models (EpiDerm and EPISKIN/SkinEthic) for testing skin corrosion (OECD TG 431). However, TG 431 does not satisfy international (GHS) labeling guidelines for transport of dangerous goods. GHS package labeling guidelines utilize 3 corrosion sub-categories (1A: very dangerous, 1B: medium danger and 1C: minor danger). Labeling a chemical as sub-category 1A has important consequences, including very small volume package limits for air transport, prohibition from passenger aircraft, protective storage conditions, costly containers and low market acceptance. Animal tests are still utilized for assessing the 1A label requirement. An *in vitro* method that discriminates 1A from 1B/1C classes will therefore have a substantial impact on reducing animal tests for this purpose. The current poster evaluates data obtained with the EpiDerm model for ability to discriminate between GHS 1A and 1B/1C classes. Data obtained from 49 chemicals tested during the ECVAM Phase I validation study plus 17 additional previously tested chemicals were retrospectively analyzed based on the MTT viability assay (50% viability cutoff) and the 3 minute exposure period. The combined set includes 15 1A, 25 1B/1C, and 26 non-corrosive chemicals. The 3 min prediction model is shown to produce a sensitivity of 93% (14/15) and overall specificity of 76% (39/51) for predicting sub-category 1A. Testing of additional chemicals (ECVAM Phase III validation study) indicates that data correction for direct MTT-reducing chemicals is important. Adoption of the 3 min EpiDerm prediction model would lead to a significant reduction in animal use for corrosion sub-group package labeling.

I-11-542

Functionality and specificity of gene markers for skin sensitization in dendritic cells

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Dendritic cells (DC) are sentinel players in this immunological cascade of skin sensitization. Using transcriptomic analyses, we recently revealed a discriminating gene expression profile in human $CD34^+$ progenitor-derived DC after exposure to skin sensitizers versus non-sensitizers. Starting from the differential expression in a small set of genes, a preliminary classification model (VITOSENS®) has been developed to identify chemicals as (non-)sensitizing in an animal-sparing approach.

The objective of the current study is to gain knowledge on the intracellular mechanism of the VITOSENS® assay. To this end, we investigated the role of the markers in the DC maturation process, and compared their activation profile by a skin sensitizer *versus* a non-sensitizing danger molecule.

To evaluate the functional relevance of VITOSENS® biomarkers in DC maturation, their response induced by the sensitizer dinitrofluorobenzene (DNFB) was pharmacologically counteracted. Flow cytometry analyses revealed that CD86 was down-regulated after COX2 inhibition, whereas expression of HLA-DR was reduced by stimulating CCR2. When exposing DC to DNFB *versus* lipopolysaccharide S (LPS), expression of the most discriminating genes, CREM and CCR2, was not altered by LPS as opposed to DNFB.

To summarize, the observations in this research indicate that a selection of the VITOSENS® genes may be functionally involved in the intracellular pathway of sensitizer-induced DC activation. By comparing their responsiveness towards a non-sensitizing danger signal and a sensitizer, VITOSENS® gene markers CREM and CCR2 appear to display a specific response.

I-11-548

In vitro assessment of skin irritation potential of surfactant-based formulations using 3D skin-reconstructed tissues and cytokine expression analysis

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The skin compatibility and safety of surfactant-based cleansers is critical in personal care products. It is desirable to minimize the dermal irritation and disruption of the skin barrier that can be caused by surfactants. We find that reproducible *in vitro* systems can accurately assess the irritation potential of the products, thus avoiding the use of animal testing. The three-dimensional EpiDerm™ model (MatTek Corp.) provides a testing platform for skin irritation assessment.

The potential dermal irritation of over 150 amphoteric and/or anionic surfactant systems was evaluated by MTT viability and IL-1α release. Diluted to 10% in water, formulations were applied onto the surface of 3D tissues for 1 h, followed by 24 h post-exposure analysis for cytokine expression. Transepidermal water loss (TEWL), the flux of water through the skin, is used clinically to assess skin barrier impairment following topical application; increased TEWL is indicative of an impaired skin barrier. An exaggerated patching model on subjects with impaired barrier due to atopic dermatitis was used to clinically assess formulation impact on skin barrier function. A correlation is found between the *in vitro* assay and *in vivo* clinical results. Also, the structure of the hydrophobic tail group of surfactant is observed to be important to surfactant mildness. The IL-1α release from coco betaine was significantly greater than that of cocamidopropyl betaine, despite both surfactants having the same hydrophilic head group. Likewise, sodium lauroyl methyl isethionate was significantly more irritating than sodium cocoyl isethionate, a mild surfactant.

I-11-567

Evaluation of the murine local lymph node assay (LLNA) for potency categorization of chemicals causing allergic contact dermatitis in humans

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ICCVAM and NICEATM jointly evaluated the usefulness and limitations of the LLNA to determine potency categorization of chemicals that may cause allergic contact dermatitis. The dose per unit skin area that induces a 5% positive response rate in the human maximization test or human repeat-insult patch test was used as the human induction threshold. Substances with induction thresholds ≤500 $\mu g/cm^2$ were classified as "strong" human sensitizers. The extent to which the LLNA EC3 (estimated concentration expected to produce a stimulation index of 3) correctly categorizes strong human sensitizers was evaluated using 136 substances with both LLNA and human data. Using EC3 ≤2%, the criterion adopted by the GHS, correctly categorized 52% (14/27) of the strong human sensitizers. However, nearly half (48% [13/27]) of the strong human sensitizers had an EC3 >2% (11/27) or were negative in the LLNA (2/27). ICCVAM concludes that the LLNA can be used to categorize substances as strong sensitizers when EC3 ≤2% but cannot be used as a stand-alone assay to determine sensitization potency categories. Additional information is required to categorize substances as other than strong sensitizers when EC3 >2%. To improve the accuracy of the LLNA for identifying strong sensitizers, ICCVAM encourages the development and evaluation of integrated decision strategies that consider other relevant information such as quantitative structure-activity relationships, structural alerts, peptide reactivity, *in vitro* data, human data or experience, and existing data from similar chemicals.

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I-11-568

Evaluation of the murine local lymph node assay (LLNA) for assessing the allergic contact dermatitis hazard potential of pesticide formulations

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ICCVAM and NICEATM jointly evaluated the usefulness and limitations of the LLNA for assessing the allergic contact dermatitis hazard potential of pesticide formulations. Most of the 104 formulations evaluated are water-soluble and were tested in an aqueous vehicle (1% Pluronic L92). Of the formulations for which LLNA and guinea pig (GP) data were available for the complete formulation (n = 23), the LLNA classified 52% (12/23) as sensitizers, while GP tests classified only 13% (3/23) as sensitizers, indicating a greater sensitivity for classifying sensitizers in the LLNA. All three formulations identified as sensitizers in GP tests were also LLNA sensitizers. The LLNA identified, as sensitizer, an additional seven substances that the GP tests classified as nonsensitizer, an overprediction rate of 50% (10/20). Based on these data, ICCVAM and an international independent peer review panel recommended that the LLNA could be used for testing pesticide formulations. This recommendation was forwarded to ICCVAM member agencies, which agreed on this expanded use of the LLNA. Several agencies also indicated that they would communicate the ICCVAM recommendations to stakeholders, and encourage appropriate use. OECD TG 429, updated in 2010, reflects the results of this evaluation, which should expand the use of the LLNA for allergic contact dermatitis hazard testing, as well as reducing and refining animal use.

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I-11-574

International acceptance of the nonradioactive LLNA: BrdU-ELISA for evaluating allergic contact dermatitis hazards

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ICCVAM and NICEATM jointly evaluated the nonradioactive LLNA: BrdU-ELISA, which measures the amount of BrdU incorporation into DNA of proliferating lymphocytes as an indicator of potential allergic contact dermatitis (ACD) hazards. Accuracy was calculated by comparing results to the traditional radioactive LLNA for 43 substances using different stimulation indices (SI) as decision criteria. SI ≥1.6 generated optimal performance; the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives) and 9/11 LLNA non-sensitizers (18% [2/11] false positives). The maximum SI for the two false positives ranged from 1.6-1.9. Eighteen substances had multiple LLNA: BrdU-ELISA tests: 85% (11/13) of the LLNA sensitizers and 60% (3/5) of the LLNA non-sensitizers were concordant. Based on these results, ICCVAM concluded that the accuracy and reliability of the LLNA: BrdU-ELISA supported its use for identifying potential ACD hazards and recommended SI ≥1.6, since there were no false negatives when compared to the traditional radioactive LLNA. Additionally, when dose-response information is not required or negative results are anticipated, ICCVAM recommended using a single-dose reduced LLNA: BrdU-ELISA, thereby reducing animal use by 40%. In July 2010, the LLNA: BrdU-ELISA was adopted by OECD as Test Guideline 442B. Availability of this international test guideline will allow more institutions to take advantage of the reduction and refinement benefits afforded by the LLNA since there is no requirement for radioactive reagents, obviating the hazards associated with their use and disposal.

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I-11-575

International acceptance of the nonradioactive LLNA: DA for evaluating allergic contact dermatitis hazards

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ICCVAM and NICEATM jointly evaluated the nonradioactive LLNA: DA, which measures ATP content as an indicator of lymphocyte proliferation, for identifying potential allergic contact dermatitis (ACD) hazards. Accuracy was calculated by comparing results to the traditional radioactive LLNA for 44 substances using different stimulation indices (SI) as decision criteria. SI ≥1.8 generated optimal performance; the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives) and 9/12 LLNA non-sensitizers (25% [3/12] false positives). The maximum SI for the three false positives ranged from 1.8-2.5. Fourteen substances had multiple LLNA: DA tests: 80% (8/10) of the LLNA sensitizers and 75% (3/4) of the LLNA non-sensitizers were concordant. Based on these results, ICCVAM concluded that the accuracy and reliability of the LLNA: DA supported its use for identifying potential ACD hazards and recommended SI ≥1.8, since there were no false negatives when compared to the traditional radioactive LLNA. Additionally, when dose-response information is not required or negative results are anticipated, ICCVAM recommended using a single-dose reduced LLNA: DA, thereby reducing animal use by 40%. In July 2010, the LLNA: DA was adopted by OECD as Test Guideline 442A. Availability of this international test guideline will allow more institutions to take advantage of the reduction and refinement benefits afforded by the LLNA since there is no requirement for radioactive reagents, obviating the hazards associated with their use and disposal.

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I-11-579

Towards animal-free testing for skin sensitization: in-house validation of four methods: MUSST, h-CLAT, KeratinoSens and DPRA

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Allergic contact dermatitis is induced by repeated skin contact with an allergen. Assessment of the skin sensitizing potential of chemicals, agrochemicals, and cosmetic ingredients is crucial to define their safe handling and use. Up to now, animal tests have been used to identify skin sensitizing potential. Animal welfare as well as the 7th Amendment to the Cosmetics Directive and REACH demands animal-free alternatives. The mechanisms of induction and elicitation of skin sensitization are complex. To account for the multitude of events in the induction of skin sensitization an *in vitro* test system will consist of a battery of various tests.

Currently, we perform in-house validations of four *in vitro* assays addressing three different events during induction of skin sensitization: 1) The peptide reactivity assay (DPRA, 1) using synthetic peptides and HPLC analysis; 2) Two dendritic cell based assays on the cell lines U937 (MUSST) and THP-1 (h-CLAT) and flow cytometric detection of the maturation markers CD54 and/or CD86 (2, 3); 3) ARE-dependent gene activity in the reporter gene cell line KeratinoSens™.

We present the results of these assays with more than 40 substances of known sensitizing potential including the performance standards defined for the LLNA. The sensitivity, specificity and accuracy of individual tests were obtained by comparison to human epidemiological data as well as to data from the local lymph node assay.

I-11-580

Determination of dimethylfumarate and analogues sensitization potential in *in vivo* and *in vitro* models

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Dimethyl fumarate (DMFu) was used as a preservative in imported house furniture such as sofas, chairs but also shoes or socks. Since 2006, this product has been implicated in many cases of skin sensitization in humans. Several thousands of consumers throughout Europe were affected, presenting weak to severe symptoms. The French health authorities have planned epidemiological and biological investigations. The aim of biological assays was to evaluate DMFu and analogues' sensitizing potential using *in vivo* and *in vitro* models. Several *in vivo* reference methods are available: the guinea pig maximization test, mouse ear swelling test and the local lymph node assay (Thy-H^3-LLNA, OECD guideline 429). In accordance with the 3R rules, the latter one was chosen to evaluate sensitizing potential of DMFu and 5 analogues: 2 fumarates: diethyl fumarate (DEFu), monomethyl fumarate (MMFu) and 3 maleates: diethyl maleate (DEMa), dimethyl maleate (DMMa), dibutyl maleate (DBMa). The efficient concentration (EC_3) was determined for each product and allowed classification. Our results confirm the strong sensitizing potential of DMFu (EC_3=0.2%). In addition, they show that all tested analogues are either moderate (DBMa, DEMa) or strong (DEFu, DMMa, MMFu) sensitizers. These molecules were also tested using the h-CLAT assay (method under pre-validation at ECVAM). H-CLAT data are in agreement with LLNA; all the products are sensitizers, DMFu being the strongest. In conclusion, this study allows the precise determination of the sensitization potential of DMFu and analogues. Also, it favours the direct comparison of *in vivo* and *in vitro* approaches.