Session 5.3
Progress and needs for developing and validating alternatives for dermal toxicity testing

Poster

Contact sensitisers induce CD86 expression and apoptosis in an independent manner in U-937 cell line

Nadège Ade[1], Silvia Teissier[1], Françoise Rousset[1] and Marc Pallardy[2]
[1] L´Oréal, Life Sciences Advanced Research, Aulnay-Sous-Bois, France; [2] INSERM U461, Faculté de Pharmacie Paris-XI, Chatenay-Malabry, France

Non-animal test methods are currently developed for the identification of skin sensitisation hazard. In this context, we focused on U-937, a human myelomonocytic cell line, which exhibits a specific induction of CD86 expression following exposure to contact sensitisers in a dose-dependent manner but not to non-sensitisers. Some contact sensitisers like $NiSO_4$ and DNCB induce CD86 expression concomitantly with a decrease in cell viability. The purpose of this investigation was to determine if CD86 expression and programmed cell death are linked. We first monitored biochemical apoptotic changes after treatment with chemicals. The results showed that $NiSO_4$ and DNCB but not SDS induced externalisation of phosphatidylserine and activation of caspase-3. Z-VAD-fmk, a pan-caspase inhibitor, inhibited DNCB- but not $NiSO_4$-induced apoptosis indicating that DNCB, an organic sensitiser, but not $NiSO_4$, a non-organic sensitiser, induces apoptosis in a caspase-dependent pathway 48 hours after treatment. When apoptosis was inhibited by Z-VAD-fmk in DNCB-treated cells, CD86 expression remained unchanged. Moreover, by flow cytometric monitoring, we showed that the major U-937 cell subset that expressed CD86 following exposure to contact sensitisers is Annexin-V negative cells confirming that CD86 is induced mainly on non-apoptotic cells. In conclusion: 1) apoptosis is not a confusing factor for the evaluation of the skin sensitisation potential of a chemical on U-937 cells and 2) phosphatidylserine externalisation measurement through Annexin-V/propidium iodide co-staining is suitable for use in the development of an *in vitro* assay for the discrimination of contact sensitisers and irritants (including tensioactive agents that disrupt the membrane integrity).

Lecture

Colipa dendritic cell research projects

Pierre Aeby[1], David Basketter[2], Walter Diembeck[3], Frank Gerberick[4] Hiroshi Itagaki[5], Ian Kimber[6], Béatrice Le Varlet[7], Irene Manou[8], Marc Paye[9], Françoise Rousset[10], Joanna Rowland[11] and Hitoshi Sakaguchi[12]

[1] Cosmital (Wella), TOX, Marly, Switzerland; [2] Unilever, Safety and Environmental Assurance Centre, Sharnbrook, UK; [3] Beiersdorf, Hamburg, Germany; [4] The Procter and Gamble Company, Cincinnati, USA; [5] Shiseido, Research Centre, Yokohama-Shi, Japan, [6] Syngenta, Central Toxicology Laboratory, Macclesfield, UK; [7] LVMH, Recherche, Saint Jean de Braye, France; [8] COLIPA, Brussels, Belgium; [9] Colgate, R&D, Milmort, Belgium; [10] L'Oreal, Advanced Research, Aulnay sous Bois, France; [11] GlaxoSmithKline, Consumer Healthcare, Weybridge, UK; [12] Kao Corporation, Tochigi, Japan

The Colipa Skin Tolerance Task Force (STTF) has played an active role in promoting refined methods for *in vivo* sensitisation tests. As a logical follow up it is actively supporting the development of *in vitro* approaches. Dendritic cells (DCs) whose central role during the induction phase of skin sensitisation is well documented, were perceived as promising *in vitro* test systems. The publication of a paper describing specific *in vitro* up-regulation of IL-1β mRNA in skin DCs exposed to sensitisers convinced STTF to initiate a research project for evaluating the relevance of IL-1β mRNA expression in cultures of human DC for predicting potential sensitisers. This study confirmed that potent sensitisers selectively up-regulate IL-1β expression in approximately 50% of donors. However, there was still a need for more robust markers and for a source of homogeneous and reproducible DCs. STTF thus initiated two complementary research projects: The holistic exploration of changes in gene expression in cultured DCs exposed to contact allergens using gene microarrays and the analysis of the modulation of human myeloid cell lines phenotype and function by chemicals. The first initiative identified several candidate genes to be further investigated for their capacity to discriminate sensitisers from non-allergens. The second is demonstrating the potential of promonocytic cell lines to respond selectively to contact sensitisers through modulation of surface markers or mRNAs. To better understand the underlying biological mechanisms, STTF is now supporting an initiative for exploring intracellular signal transduction pathways in Langerhans cells during activation/maturation process induced by sensitisers.

Poster

In vitro activation of dendritic-like cells: A new tool for the elucidation of the sensitising properties of chemicals

Pierre Aeby[1], Otto Goettel[1], Heinz Beck[1], Laurent Chassot[1] and Carsten Goebel[2]*

[1] Cosmital SA (Wella AG), Marly, Switzerland; [2] Wella AG, Product Safety, Darmstadt, Germany

In vitro analysis of the maturation of dendritic-like cells (DCs) induced by chemicals is becoming a useful tool for the elucidation of the sensitising properties of chemicals. We have used this new approach to analyse the different sensitising properties of p-phenylenediamine (PPD), its oxidation products including Bandrowski's base (BB), and of p-toluylenediamine (PTD). Since acetylation is known to be a major pathway in human, mouse and rat hepatocytes as well as in human keratinocytes, mono- and di-acetylated PPD and PTD were synthesised and evaluated. Our *in vitro* test protocol uses human peripheral blood monocytes derived DCs that are exposed for 3 to 30 hours to the test chemicals. DC maturation is evaluated by flow cytometric measurement of the percentage of CD86 positive cells and quantitative measurement of the mRNA expression of interleukin-1β, interleukin 8 and aquaporin P3 using the Lightcycler® real time PCR system. Fresh PPD induces only a slight DC maturation whereas oxidised PPD and BB are much more potent inducers. On the other hand, fresh PTD is a potent inducer and does not need a prior oxidisation step. We could also show for the first time that mono- and di-acetylated PPD or PTD do not induce any relevant DC maturation. *In vivo* results obtained with the same chemicals in the murine local lymph node assay confirmed the *in vitro* findings. We conclude that the described *in vitro* test system allows a refined analysis of the sensitising properties of chemicals and will further improve product safety.

Poster

The COLIPA *strategy* for the development of *in vitro* alternatives: Skin sensitisation

David Basketter (Chairman)[1], Pierre Aeby[2], Walter Diembeck[3], Frank Gerberick[4], Hiroshi Itagaki[5], Ian Kimber[6], Béatrice Le Varlet[7], Irene Manou[8], Marc Paye[9], Françoise Rousset[10], Joanna Rowland[11] and Hitoshi Sakaguchi[12]

[1] Unilever, Safety and Environmental Assurance Centre, Sharnbrook, UK; [2] Cosmital SA (Wella AG), Marly, Switzerland; [3] Beiersdorf, Hamburg, Germany; [4] The Procter and Gamble Company, Cincinnati, OH, USA; [5] Shiseido Research Centre, Yokohama-Shi, Japan; [6] Syngenta, Central Toxicology Laboratory, Macclesfield, UK; [7] LVMH Recherche, Saint Jean de Braye, France; [8] COLIPA, Brussels, Belgium; [9] Colgate-Palmolive, Liege, Belgium; [10] L'Oréal, Advanced Research, Aulnay sous Bois, France; [11] GlaxoSmithKline, Surrey, UK; [12] Kao Corporation, Tochigi, Japan

Skin sensitisation represents the allergic activation of the immune system in response to repeated exposure to a subset of chemicals with the ability to behave as haptens, i.e. they can covalently bind to skin proteins. The clinical expression of skin sensitisation is allergic contact dermatitis, whose symptoms (e.g. erythema, oedema) are often similar to those of skin irritation. Historically, guinea pig models allowed the identification of potential skin sensitising chemicals. More recently, a refined, reduced method, the murine Local Lymph Node Assay (LLNA) has been employed. Our present aim is to undertake the work necessary to ensure the final step, replacement of animal testing, can be achieved. To this end, the COLIPA Skin Tolerance Task Force (STTF) has undertaken a range of research projects, from aspects of chemistry/peptide binding/skin metabolism, through evaluation of intracellular signalling pathways induced by allergens, to allergen induced changes in dendritic/Langerhans cells measured at genomic and protein level. The knowledge gained from this work aims to develop and pre-validate *in vitro* predictive assays. The current challenge is developing an appreciation of how to use their data output for risk assessment in addition to simple hazard identification.

Lecture

Use of *in vitro* data for sensitisation risk assessment

David Basketter

Unilever – Safety and Environmental Assurance Centre, Colworth Laboratories, Bedfordshire, UK

The predictive identification of chemicals which possess the intrinsic ability to cause skin sensitisation represents only the very first step in the safety assessment of this toxicity endpoint. Once a sensitiser has been identified it is then necessary to determine whether there are conditions under which skin exposure may occur without the risk of inducing skin sensitisation. This determination is made by a risk assessment process, which historically involved a comparative approach, largely based on assessments of similarity of allergens in predictive guinea pig tests. More recently, a Quantitative Risk Assessment (QRA) approach has been promulgated, which depends on the characterisation of the relative potency of a sensitiser in the Local Lymph Node Assay (LLNA). In this assay, an objective quantitative measure of potency is used as the basis for the calculation of safe exposure levels in different product settings. Thus, a key challenge for the *in vitro* methods which will replace assays such as the LLNA is how the data derived from them can be utilised not only to identify a skin sensitiser, but also to characterise its relative potency. Thus as we develop *in vitro* alternatives for skin sensitisation, we must examine how to quantify the output from such methods and determine how to integrate multiple endpoints in a consistent and transparent manner such that a prediction of human skin sensitisation potency can be achieved.

Poster

ECVAM key area on sensitisation: Summary of ongoing activities

Silvia Casati
ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, Italy

Assessing the sensitising potential of substances represents one of the major issues in the context of toxicological evaluation for the protection of human health, especially in view of the recent increase in the prevalence of skin and respiratory allergies. Although a number of promising *in vitro* and *in silico* systems are under investigation, animal tests are still currently used for the identification of chemical allergens. The primary objective of this key area is to develop and validate alternative testing strategies to replace animal experimentation. A first skin sensitisation Task Force meeting was organised in 2003 (chair D. Basketter) to advise ECVAM on future activities to be undertaken in this field to complement, to support and to harmonise ongoing efforts in the area. Following the recommendations of the Task Force, in 2004 a workshop was organised to review the state of the art of the use of cultured dendritic cells for the identification of skin sensitisation hazard (ATLA, Casati et al., 2005). Subsequently ECVAM took the lead together with COLIPA in the conception of an Integrated Project "Sens-it-iv" which has recently been evaluated and accepted by DG RTD. The overall goal of Sens-it-iv is to develop, with different partners, over a period of five years, strategies to replace animal experimentation with *in vitro* assays for identifying skin and respiratory sensitisers for chemical, cosmetic and pharmaceutical substances. The *in vitro* assays to be developed will allow the testing of the sensitising potency for classification and labelling and for the purpose of risk assessment.

Lecture

Hapten-protein binding – what do we know?

Maja Divkovic
Safety and Environmental Assurance Centre, Unilever Colworth, Bedford, UK

The search for alternatives to *in vivo* testing of chemicals for skin sensitisation hazard and potency is dependent on improving our understanding of the molecular events. The sensitising potential of a chemical is directly linked to its reactivity towards proteins. Known sensitisers are either directly capable of covalently modifying proteins or are metabolised into protein reactive species. Additionally, small organic compounds are unable to induce a significant immune response and thus need to form a macromolecular immunogen. A protein reactive chemical could covalently modify any available nucleophiles given the suitable conditions but the immunological relevance of the particular modifications is unknown. Therefore the use of model proteins/peptides in such investigations is necessary. The data obtained from a limited number of studies demonstrate that the covalent protein binding is a characteristic of tested sensitisers. The three dimensional protein environment may restrict chemical modifications as only some of numerous reactive side chains are modified. Model peptide studies often produce different results with the same chemicals. Whilst such investigations further aid our understanding of the protein–hapten interactions, the knowledge about immunogenic relevance of observed modifications is sparse. Better model proteins/peptides could be chosen and better predictive assays designed if particular types of modifications were shown to be more relevant than others. Understanding protein–hapten binding mechanisms will increase the confidence in prediction of sensitisation hazard using *in silico* tools, as well as provide the opportunities to develop simple, accurate and cost effective predictive *in vitro* assays for skin sensitisation hazard and potency.

Lecture

Designing practical protein binding assays for screening skin allergens

Frank G. Gerberick
Procter and Gamble Company, Central Product Safety, Cincinnati, USA

There are a variety of characteristics that determine whether a chemical can function as a skin allergen including the ability to penetrate into the skin, react with protein, and be recognised as antigenic by immune cells. Since protein reactivity is a key step in the induction of skin sensitisation it is hypothesised that reactivity could be used to screen for the sensitisation potential of chemicals. Chemical allergens are electrophilic and as such react with nucleophilic amino acids like cysteine or lysine. The research aim is to develop an *in vitro* peptide reactivity method that allows for quantitative analysis of a chemical's reactivity potential. In order to determine if reactivity correlates with sensitisation potential, work is underway in various laboratories to evaluate peptides containing the best nucleophiles for evaluating the reactivity of allergens and non-allergens. To date, excellent progress has been made demonstrating a significant correlation between a chemical's skin sensitisation potency and its ability to react with peptides containing nucleophilic amino acids such as cysteine and lysine. Current work is focused on incorporating a metabolism system in the assay so that pro-haptens can also be identified and categorised as well as adapting the method to a high-throughput format for screening large numbers of chemicals. With the development of a robust chemical reactivity assay it is hoped that it will be possible to screen new chemicals *in vitro* and thus reduce the reliance on animal test methods.

This research is supported by the European Cosmetic, Toiletry and Perfumery Association (COLIPA).

Poster

Identification of potential dendritic cell markers for the prediction of skin sensitisation using real-time PCR

Lucy Gildea[1], Cindy Ryan[1], Jennifer Kennedy[1], Leslie Foertsch[1], Rebecca Dearman[2], Ian Kimber[2] and Frank Gerberick[1]*
[1] The Procter and Gamble Company, Central Product Safety, Cincinnati, OH, USA;
[2] Syngenta Central Toxicology Laboratory, Macclesfield, UK

Changes that occur within epidermal dendritic cells after allergen exposure represent potential endpoints for predictive *in vitro* skin sensitisation methods. Previous microarray analysis of human Peripheral Blood-derived Dendritic Cells (PBMC-DC) revealed changes in gene expression following dinitrobenzene sulfonic acid (DNBS)-allergen treatment. Using a select group of chemicals, the sensitivity, selectivity, and dynamic range of those genes changes were evaluated by quantitative PCR to determine their usefulness as markers for contact allergy. From that work a focused candidate gene list was selected. Subsequent validation of the target genes was performed using an expanded chemical dataset. PBMC-DC were treated for 24 hours with various doses of chemicals. RNA was extracted and used in real-time PCR reactions using specific primers. Mean Relative Fluorescence Units (RFU) were calculated and then converted to mean fold changes comparing mean RFU in control (vehicle-treated) samples *versus* mean RFU in treated samples. The dynamic range and sensitivity of these genes were evaluated by testing multiple doses of sensitisers of varying allergenic potencies, such as hydroxycitronellal and hydroquinone, as well as chemical irritants. As a result of that work, approximately 20 potential genes have been identified that meet the selection criteria for sensitivity, dynamic range, and reproducibility. Some of the allergen-induced changes were in the expression of genes associated with immune function, such as CCL2, CCL4, CCL23, SLAM, and the Lectin Receptor. These genes will be analysed further for their usefulness as an endpoint measure for the prediction of contact allergy.

This work was funded in part by COLIPA.

Poster

Cytokine secretion profiles of mouse dendritic cells (DC): Influence of cell injury

Ian Kimber[1], Catherine Betts[1], Gemma Beckwith[1], Laura Bailey[1], Cindy Ryan[2], Frank Gerberick[2] and Rebecca Dearman[1]

[1] Syngenta CTL, Macclesfield, UK; [2] Proctor and Gamble, Cincinnati, USA

Cutaneous immune responses such as chemical contact allergy are regulated by cytokines expressed by keratinocytes and Langerhans cells (LC). Bone marrow derived LC-like DC were generated from BALB/c strain mice and cytokine secretion profiles measured following 24 h culture with the water soluble allergen dinitrobenzene sulfonic acid (DNBS) and the non-sensitising analogue, benzene sulfonic acid (BSA). Concentrations of DNBS or BSA were utilised that were without effect on cell viability, or that provoked significant cytotoxicity (measured by trypan blue exclusion). Control cells were cultured with medium alone, or with 1 μg/ml bacterial lipopolysaccharide (LPS), a potent stimulator of DC. DC expressed constitutively interleukin (IL)-1β, IL-1β, IL-6 and IL-12. Culture of DC with 1μg/ml LPS resulted in very marked (20 to 70fold) increases in expression of all cytokines. Culture with concentrations of DNBS (5 and 1 mM) that resulted in greater that 50% cytotoxicity reduced cytokine expression. Treatment with 0.5 mM DNBS, a non-toxic dose, stimulated modest increases in IL-6 and IL-12 secretion. In contrast, culture with cytotoxic doses of BSA (100 and 50 mM) provoked low level increases in IL-1β, IL-1β and IL-6, with a concomitant decrease in IL-12 production. These data suggest that it may be possible to distinguish chemical allergens from irritants on the basis of differential cytokine secretion under conditions of equivalent cell injury.

Poster

Differences in surface marker expression on monocyte derived dendritic cells induced by LPS, irritants, and contact allergens

Ludger Kolbe, Jochem Spieker, Silke Gerlach, Horst Wenck and Walter Diembeck

Beiersdorf AG, R&D, Hamburg, Germany

Dendritic cells play a pivotal role in the induction of skin allergies. After contact with sensitisers these cells undergo rapid maturation and migrate to the local lymph node in order to activate allergen specific T cells. Dendritic cell maturation is characterised by changes in cell surface marker expression and increased cytokine production. These changes represent potential endpoints for predictive *in vitro* skin sensitisation assays. In recent years much progress has been made in developing *in vitro* methods to study allergen induced changes in dendritic cells. However, it will still take some effort to develop a reliable *in vitro* method for the identification of potential skin sensitisers. Robust markers are required to distinguish potential allergens from irritants and innocuous substances. For this purpose we studied the time course and dose response relationship of allergen induced surface marker expression on monocyte derived dendritic cells. We determined changes in CD80, CD86, HLA-DR, CD83, and CD54 expression on dendritic cells after incubation with allergens of varying potency by flow cytometry. Lipopolysaccharide, Triton-x100, and SDS were used as irritant control substances. Our test design allows to discriminate between weak sensitisers and irritants and our data also demonstrate that test concentrations must be chosen carefully. High concentrations of sensitisers may result in low cell viability and therefore may give false negative test results.

Lecture

The chemistry of skin allergy

Jean-Pierre Lepoittevin
Laboratoire de Dermatochimie, Clinique Dermatologique, CHU, Strasbourg, France

Allergic Contact Dermatitis (ACD) is a very common disease resulting from epidermal proteins being chemically modified by haptens. The processing of such modified proteins by Langerhans cells, the main antigen-presenting cells in the epidermis, generates altered peptides that are subsequently presented, in association with MHC molecules, to naive T-lymphocytes in the lymph nodes. The whole process results in the selection and activation of T-lymphocyte sub-populations with T-cell Receptors (TcR) specific for the chemical modification. Haptens are usually low molecular weight molecules, lipophilic enough to penetrate the epidermis through the *stratum corneum*, and with a potent chemical reactivity allowing the formation of a covalent link with nucleophilic residues on protein amino acid side chains. For some time it has been considered that the more a molecule was able to modify proteins, the better a sensitiser was and that a direct relation could be established between the overall chemical reactivity of a molecule and its sensitising potential. Today, it is hypothesised that the sensitising potential of a molecule is related to its chemical reactivity towards a few specific amino acids relevant to the sensitisation process. Haptens can modify proteins through many different mechanisms, from classical nucleophilic-electrophilic reaction to radical reactions. The knowledge of how haptens can modify proteins is the base for the development of predictive alternative tests aimed at the identification of hazard and potency such as Structure Activity Relationships (SAR), Quantitative Structure Activity Relationships (QSAR) and peptide reactivity tests.

Poster

Predicting the classification of skin sensitisation potency using 4D-fingerprints and logistic regression with and without partial least-squares

Yi Li[1], Anton J. Hopfinger[1], Petra S. Kern[2] and Frank G. Gerberick[3]*

[1] University of Illinois at Chicago, Laboratory of Molecular Modeling and Design, College of Pharmacy, Chicago, USA; [2] Procter and Gamble Eurocor, Strombeek-Bever, Belgium; [3] The Procter and Gamble Company, Miami Valley Innovation Center, Cincinnati, OH, USA

A set of 132 structurally diverse compounds whose skin sensitisation potencies are none or weak (class 1), and strong or extreme (class 2) formed a training set to build two-state categorical QSAR models. A test set of 15 compounds spanning the structural diversity of the training set was used to evaluate the categorical QSAR models. Skin sensitisation potency measures were taken from historic local lymph nodes assay data. 4D-fingerprint descriptors were employed to construct the models. Model fitting was performed using Logistic Regression (LR) with, and without, Partial Least-Squares (PLS). The training set cross-validated accuracy of prediction of the LR models without PLS ranges from 77.3% to 78.0%. For PLS-LR models the accuracy spans 87.1 to 89.4%. The corresponding test set prediction accuracies range from 87.1 to 89.4% for LR and 73.3 to 80.0% for PLS-LR models. The categorical QSAR models are composed of descriptor terms related to aromatic atoms, hydrogen bond acceptor groups and negatively partially charged atoms. The descriptors of the models also define molecular size and shape features for skin sensitisation potency. The 4D-fingerprint LR, and PLS-LR, models developed in this study are superior in both data fit and predictivity to alternate models developed by ourselves, and models proposed by others, which use different descriptors, including classic 2D QSAR descriptors, and/or other data fitting methods.

Senese, C. L., Duca, J. and Pan, D. et al. (2004). 4D-fingerprints, Universal QSAR and QSPR descriptors. *J. Chem. Inf. Comput. Sci. 44*, 1526-1539.

Poster

Investigation of co-stimulatory molecule expression and cytokine production on THP-1 human monocyte cell line treated with skin sensitisers

Makoto Mizuno, Noriyasu Imai, Yoshikazu Naoe, Miki Nakanishi and Yuko Okamoto
KOSÉ Corporation, Fundamental Research Center, Tokyo, Japan

Dendritic Cells (DCs) play a critical role in the induction phase of sensitisation by presenting antigens. It has been demonstrated that the application of chemical hapten to DCs induces the production of some inflammatory cytokines and up-regulates the expression of class II major histocompatibility complex (MHC class II) and co-stimulatory molecules. Therefore, DCs have been used for the development of *in vitro* sensitisation test method. Recently, various methods using human monocytic cell lines as a substitute for DCs have been progressed. In this study, we cultivated THP-1, a human monocyte cell line, with skin sensitisers or non-sensitisers, and evaluated the production of cytokines and the expression of co-stimulatory molecules on THP-1 to develop *in vitro* skin sensitisation test method. The culture supernatants were collected and the production of cytokines was measured by ELISA. The expression of CD86 and CD54 on THP-1 was measured by flow cytometry. Our results indicated that treatment with sensitisers such as 2,4-dinitrochlorobenzene or nickel sulfate, but not non-sensitisers, induces the production of TNF-α and IL-1β and up-regulates the expression of CD86 and CD54. This study suggests that measuring cytokine production and co-stimulatory molecule expression on THP-1 is a useful *in vitro* method to predict skin sensitisation potential of chemicals.

Poster

Evaluation of the peptide-binding assay by using LC-mass spectrometry as a skin sensitisation test

Yosuke Nakamura[1], Kazuko Yamashita[2], Takashi Morimoto[1], Tamaki Higaki[1], Hiroshi Kato[1], Masahiko Okamoto[2], Mika Ota[1] and Satoshi Kawamura[1]

[1] Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory, Osaka, Japan; [2] Sumitomo Chemical Co., Ltd., Organic Synthesis Research Laboratory, Osaka, Japan

Allergic contact dermatitis is a common occupational health hazard for workers handling a lot of chemicals. To protect the workers from this hazard, it is necessary to assess the skin sensitisation potential of not only the final product but also the intermediates in a manufacturing process. But the number of them is so large that it is practically impossible to check the sensitisation potential of all of them by using a local lymph node assay. Thus, it is important to develop the short-term alternative methods for evaluation of the skin sensitising potential of the chemicals. As the majority of the skin sensitisers bind covalently to amino acids such as cysteine or lysine to provoke the immunologic reactions, it may be useful to check the binding ability of the chemicals to a peptide such as glutathione. To evaluate the correlation between the results of the peptide-binding assay and the animal tests, the peptide-conjugate formation of 82 chemicals, including 61 sensitisers and 21 non-sensitisers, was analysed by LC-mass spectrometry. Based on the structural analysis, it was concluded that 30 out of 61 sensitisers showed the peptide-conjugate formation but 19 out of 21 non-sensitisers did not in this study. Thus, the total concordance rate was 60% and the predicted performance for the *in vivo* test was 94%. Our data show the peptide-binding assay by using LC-mass spectrometry is a good alternative method as a screening test for the evaluation of the skin sensitisation potential of the chemicals.

Lecture

Collaborative study for the preparation of a test protocol based on the U937 human cell line for predicting skin sensitisation potential

Jean-Marc Ovigne[1], Françoise Rousset[1], Pierre Aeby[2], François Python[2] and Béatrice Le Varlet[3]*

[1] L´Oréal, Life Sciences Advanced Research, Aulnay-sous-Bois, France; [2] Cosmital SA (Wella AG), Marly, Switzerland; [3] LVMH, Recherche, Saint Jean de Braye, France

One of the alternatives to animal testing for the prediction of skin sensitisation is *in vitro* methods based on the use of human monocytic cell lines as surrogate dendritic cells. The European Cosmetic, Toiletry and Perfumery Association (COLIPA) is supporting ring trials to shed light on the practicability of this approach. Among the different human cell lines tested, the pro-monocytic cell line U937 has been identified as a good candidate responding to chemical sensitisers. The following U937/CD86 test protocol based on the up-regulation of CD86 expression, a co-stimulatory molecule, by U937 cells after 48 h exposure to chemicals has been proposed for pre-evaluation and further optimisation. Expression of CD86 is assessed by flow cytometry and a test item is declared positive if: i) viability (PI exclusion) is at least 70%, ii) CD86 expression reaches ≥ 120% of control cells (EC120, the concentration at which this happens, is calculated by linear regression), iii) the chemical induces CD86 in a dose dependent manner, iv) there are 2 out of 3 concordant experiments. Similar preliminary results were obtained by three independent laboratories (L'Oréal, Cosmital and LVMH) with two sensitisers (nickel sulfate and dinitrochlorobenzene) and one irritant (sodium dodecyl sulfate) indicating that upon further improvement of the culture conditions and test parameters, the U937/CD86 test system could be the basis for the development of an *in vitro* test system. A ring trial co-ordinated by the COLIPA should follow with a panel of reference sensitisers and irritants to evaluate the U937/CD86 test.

Poster

In vitro prediction of the skin sensitisation potential of chemicals using the U937/CD86 test

Jean-Marc Ovigne, Denis Verda, Cécile Piroird and Françoise Rousset

L´Oréal, Life Sciences Advanced Research, Aulnay-Sous-Bois, France

Among the alternatives to animal testing for the prediction of skin sensitisation is *in vitro* methods based on the use of human monocytic cell lines as surrogate dendritic cells. One of the different human cell lines tested, the pro-monocytic cell line U937 has been identified as a good candidate responding to chemical sensitisers.

L'Oréal has developed the U937/CD86 test based on the up-regulation of CD86 expression, a co-stimulatory molecule, by U937 cells after 48 h exposure to chemicals. Expression of CD86 is assessed by flow cytometry and a positive result is recorded if: i) viability (PI exclusion) is at least 70%, ii) CD86 expression reaches 120% of control cells, iii) the chemical induces CD86 in a dose dependent manner, iv) there are 2 out of 3 concordant experiments. A wide concentration range is first tested and then refined based on cell toxicity and CD86 positivity. When all criteria are fulfilled, EC120, the concentration at which CD86 reaches 120% is calculated by linear regression.

With a panel of ~70 reference chemicals (sensitisers and non sensitisers), a correlation of 95% with the human clinic was observed for the prediction of the sensitising potential using the U937/CD86 test.

A ring study for inter-laboratory evaluation of the U937/CD86 test is about to start (see theme 5, session 3) with the support of the European Cosmetic, Toiletry and Perfumery Association (COLIPA).

Lecture

Results of a ring trial of a human Cell Line Activation Test (h-CLAT) for predicting skin sensitisation potential

Cindy Ryan[1], Hitoshi Sakaguchi[2], Takao Ashikaga[3], Françoise Rousset[4], Klaus Schroeder[5] and David Basketter[6]

[1] The Procter and Gamble Company, Central Product Safety, Cincinnati, Ohio, USA; [2] Kao Corporation, Safety and Microbial Control Research Center, Tochigi, Japan; [3] Shiseido Co., Ltd., Safety and Analytical Research Center, Kanagawa, Japan; [4] L'Oréal, Advanced Research, Aulnay, France; [5] Henkel, Dusseldorf, Germany; [6] Unilever, Safety and Environmental Assurance Centre, Sharnbrook, UK

In vitro methods based upon changes in cell surface marker expression induced in DC following exposure to contact allergens represent one approach for developing non-animal test methods for skin sensitisation. Donor to donor variability in response to allergen exposure has been observed in DC derived from human peripheral blood. Therefore, methods which utilise human monocytic cell lines as surrogate DC are being explored. The human Cell Line Activation Test (h-CLAT) examines the level of expression of CD86, a co-stimulatory molecule, and CD54, an inter-cellular adhesion molecule, on the surface of THP-1 cells (monocytic leukemia cell line) using flow cytometry following 24 hours of chemical exposure. The h-CLAT was evaluated by five independent laboratories in a ring trial co-ordinated by the European Cosmetic, Toiletry and Perfumery Association (COLIPA). A total of ten chemicals were evaluated in two separate trials and included seven sensitisers covering a range of allergenic potencies and three irritants. All laboratories correctly identified six of the seven known allergens as sensitisers and two of the three irritants as non-sensitisers. α-Hexylcinnamic aldehyde, an allergen of moderate potency, was classified as a sensitiser by only two of the five labs. False positive results were obtained with the irritant benzalkonium chloride by two labs. As a consequence of the inter-laboratory differences observed in this trial, opportunities for refinement of the h-CLAT protocol and prediction model were identified. Additional work is needed to support the feasibility of utilising cell lines as surrogate DC in development of *in vitro* skin sensitisation methods.

Poster

Use of historical local lymph node data in the development of alternative test methods for skin sensitisation

Cindy Ryan[1], Petra Kern[2], David Basketter[3], Grace Patlewicz[3], Rebecca Dearman[4], Ian Kimber[4] and Frank Gerberick[1]

[1] The Procter and Gamble Company, Central Product Safety, Cincinnati, OH, USA; [2] The Procter and Gamble Company, Central Product Safety, Brussels, Belgium; [3] Unilever, Safety and Environmental Assurance Centre, Sharnbrook, UK; [4] Syngenta Central Toxicology Laboratory, Macclesfield, UK

In developing new alternative test methods, the availability of high quality, relevant and reliable *in vivo* data for the endpoint of interest is essential. Data derived in humans would be the most appropriate as test methods attempt to predict a toxicological effect in man. However, a sufficient quantity of such data is most likely not available, so data derived from animal studies usually serves as the basis for comparison. Recently, the Local Lymph Node Assay (LLNA) has emerged as a practical option for assessing the skin sensitisation potential of chemicals. In addition to accurately identifying sensitisers, the LLNA has also been shown to provide a reliable measure of relative sensitisation potency; information critical to the successful management of human health risks. Therefore, for use in evaluating new test methods for skin sensitisation and the development of quantitative structure-activity relationship models, a database of historical LLNA data for 232 different chemicals has been created. This extensive dataset encompasses the biological and chemical diversity of known skin allergens. The range of relative allergenic potencies are represented in the dataset by 18 extreme, 31 strong, 76 moderate, and 65 weak contact allergens as well as 42 non-sensitising chemicals. Aldehydes, ketones, aromatic amines, quinones and acrylates are among the diverse chemical classes represented in the dataset. In addition to 2D chemical structures, the physicochemical parameters included are logKp, logK and molecular weight. It is hoped that this database will accelerate the development, evaluation and eventual validation of new approaches to skin sensitisation testing.

Poster

Evaluation of the MUTZ-3 cell line as a model system for *in vitro* testing of sensitisation

Elke Schoeters[1], Geert Verheyen[1], Rosette Van Den Heuvel[1], Hilda Witters[1], Inge Nelissen[1], Zwi Berneman[2], Vigor Van Tendeloo[2] and Greet Schoeters[1]

[1] VITO, Environmental Toxicology, Mol, Belgium; [2] University of Antwerp, Hematology, Antwerp, Belgium

The early detection of the sensitising potential of chemicals is of great importance to the industry. The number of animals that are currently used for these experiments has to be reduced, so *in vitro* alternatives are necessary. A promising alternative is an *in vitro* test system based on a model of human Dendritic Cells (DC), cultured from CD34+ progenitors. However, this DC model has several drawbacks. For instance, human cord blood has to be collected/available to obtain CD34+ stem cells. Furthermore, primary DC cultures are time-consuming, and experiments are hampered by the inter-individual differences that exist between donors. MUTZ-3 is a human, acute myeloid leukemia cell line with the potential to differentiate into immature DC and may therefore be used to overcome many of these difficulties.

We optimised the culture conditions of the MUTZ-3 cell line, allowing stable propagation of naive cells and optimal differentiation into immature DC. The phenotypes of the naive cells and immature DC were characterised by means of the expression of relevant surface markers. To further characterise the MUTZ-3 cell line, both naive cells and MUTZ-3-derived immature DC were exposed to maturation stimuli, such as LPS, and the maturation response was analysed by flow cytometry. To preliminarily evaluate the impact of sensitising chemicals on maturation of MUTZ-3-derived DC, immature DC were exposed to different concentrations of nickel sulphate and DNCB. Additional experiments will be required to reveal whether the MUTZ-3 cell line is a valid model system for *in vitro* testing of sensitisation.

Poster

Contact sensitisers induce phosphorylation of p38 MAPK in U937 cells; a possible marker to discriminate between sensitising potential and biological activity?

Silvia Teissier, Denis Verda, Jean-Marc Ovigne and Françoise Rousset

L'Oréal, Life Sciences Research, Aulnay sous Bois, France

Contact sensitisers induce several phenotypic and functional changes of dendritic cells (DC) *in vivo* and *in vitro*. One of these changes, the induction of CD86, is the most frequently analysed endpoint for the *in vitro* prediction of contact sensitisers using different cellular models based on DC or human myeloid cell lines. This marker has proven its relevance to evaluate the sensitising potential of chemicals. In contrast, in the context of pharmacophores, the evaluation of the sensitising potential may be problematical, since these bioactive molecules may interfere with the cellular processes involved in the induction of cell maturation and differentiation measured by the CD86 expression. In order to anticipate on this issue, we decided to explore very early events of the signal transduction activated by contact sensitisers in our U937 based model, which finally results in the induction of CD86. In this study we analysed the phosphorylation of p38 MAPK induced in U937 cells after treatment with several sensitisers, irritants and bioactive molecules. As already showed for DC, we confirmed in our U937 model, that contact sensitisers specifically activate p38 MAPK. Moreover, preliminary results indicate that phosphorylated p38 is a promising marker for the discrimination between biological activity and sensitising potential of a molecule.

Poster

NF-κB, PI3K and JNK are essential signalling pathways implicated in human dendritic cells maturation after contact with allergens

Sandra Trompezinski[1], Julie Denoeud[2], Béatrice Zabawski[2], Claude Vincent[2], Daniel Schmitt[2], Pascal Courtellemont[3], Béatrice Le Varlet[3] and Mireille Serres[2]

[1] UCBL1 EA37-32 and European Cosmetic Toiletry and Perfumery Association (COLIPA), Dermatology, Lyon/Bruxelles, France/Belgium; [2] UCBL1 EA37-32, Dermatology, Lyon, France; [3] LVMH, Recherche, St Jean de Braye, France

A critical step in the initiation of allergic reaction is the activation of naive T cells by Dendritic Cells (DC). *In vitro*, various agents are well-known to induce DC maturation, such as LPS, TNF_ and contact sensitisers. This process is essentially characterised by an increase in cell surface expression of CD86, HLA-DR and CD54 and by cytokine production. However, only few studies have been reported on the intracellular mechanisms by which human DC respond to allergen treatments. A better knowledge of the kinases activated in this process is required for the understanding of immune response induced by allergens. For this purpose, our studies were performed on DC derived from human monocytes after 5 days of culture with GM-CSF and IL-4. Then, immature DC were induced to maturate by nickel or LPS and TNFα (positive controls) for 48 h in X-VIVO medium without serum. To study the main kinases implicated in MAPKs (Erk 1/2, p38 MAPK and JNK), PI3K, PKC, PKA and NF-κB pathways, specific inhibitors at each step of phosphorylation cascades were used.

Our data demonstrated that CD86, CD54 and HLA-DR expression induced by DC maturation was essentially suppressed by inhibitors of JNK, PI3K, PKCσ and NF-κB pathways, suggesting a major role of these kinases. PKA does not seem to be directly implicated and the role of Erk1/2, p38 MAPK and other PKC isoforms remain to be specified. These studies will be extended to other allergens and irritants in perspective to develop alternative methods for contact dermatitis prediction.

Poster

Construction of three-dimensional human skin model consisting of dendritic cells, keratinocytes and fibroblasts

Tadashi Uchino and Hiroshi Tokunaga

National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo, Japan

In order to evaluate the immunoreaction on human skin, we attempted to the three-dimensional human skin model consisting of three different cells such as dendritic cells, keratinocytes and fibroblasts. Checking the histological cross-section of the human skin model, we stained the human skin model with hematoxylin and eosin. After 11-day incubation the homy layer was initially observed and then 14-day incubation three-dimensional human skin model was completely formed. Due to non-cytotoxic dose of DNCB, $NiSO_4$ and compound48/80, the dendritic cells in the human skin model released IL-4, IL-2 and IL-1α into the incubating medium and expressed CD86. The results suggest that the three-dimensional human skin model with dendritic cells should be able to apply for studying the effect of immune-sensitising compounds.

Poster

Development of a peptide reactivity prediction model for screening the skin sensitisation potential of chemicals

Jeff Vassallo[1], Frank G. Gerberick[1], Joel Chaney[1], Ruth Bailey[1], Steve Morrall[1], Brad Price[1] and Jean-Pierre Leopoittevin[2]*

[1] The Procter and Gamble Company, Central Product Safety, Cincinnati, USA; [2] Université Louis Pasteur, Laboratorie de Dermatochimie, Strasburg, France

In order for a chemical to function as a contact allergen it must penetrate the skin, react with protein and be recognised as antigenic by immune cells. It is well established that protein reactivity is associated with skin sensitisation potential. An *in vitro* assay using peptides containing cysteine or lysine, or glutathione as surrogate nucleophiles was recently developed and used to measure the reactivity of more than sixty chemicals at various concentrations with sensitisation potencies ranging from weak to strong, and non-sensitisers. Using peptide reactivity data, in addition to LLNA potency data, a prediction model based on classification tree methodology was developed. Following reactivity determination, a compound is classified as a strong sensitiser if it reacts with more than 19% of the glutathione. A second test identifies a compound as a non-sensitiser if it reacts with less than 6% of the 1:10 cysteine peptide. The remaining compounds are classified as weak or moderate depending upon whether they react with less than or more than 76% of the 1:50 cysteine peptide, respectively. Using these model predictors, the potency of additional allergens was determined following reactivity testing. Model predicted potencies were within one category of the LLNA determined potency for all chemicals tested and the model identified correctly all of the non-sensitisers. Collectively, these data indicate that this model may be useful in screening the potency of skin allergens.

This research was supported by the European Cosmetic Toiletry and Perfumery Association (COLIPA).