Next generation skin sensitisation testing

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DNA is the software that predicts human phenotypes. Genomics utilises DNA as such a piece of software, which in several recent applications has been demonstrated to be able to predict a number of biological features, enabling anything from face recognition to vaccine development.[1] The tremendous information content, harboured in the DNA, should be harnessed in tests of cosmetic ingredients and formulations, to get a complete insight into what is happening in the body when these types of products are applied to the human skin. This type of complex information gives a holistic view of a human condition that many of us are familiar with, such as e.g. allergic contact dermatitis, and has the potential not only to classify chemicals used in cosmetics but also to determine the magnitude to which a chemical affects the human body (the potency).

This distinguishes genomics from most other test principles, where in many cases only one or two markers are being monitored, such as DC activation markers (CD86, CD54),[2,3] genes involved in cytoprotective responses to oxidative stress or electrophilic compounds (Nrf2, Keap1),[4,5] or proinflammatory cytokines, (e.g. IL18).[6]

Genomics utilises the entire transcriptome of cells, tissues or organs that have been exposed to a particular chemical in order to provide insight also into what mechanisms are activated in each specific cell, tissue or organ. The transcriptome, i.e. the expressed levels of genes, are subsequently analysed to deliver the appropriate information, in this case whether or not a certain chemical compound is an allergen. There are a number of applicable genomic technologies, including whole genome microarray technology.[7,8] The measured endpoints for such a microarray are the transcribed mRNA levels of over 20,000 genes, consequently harbouring massive amounts of information, which can be deciphered in silico into knowledge by bioinformatics, focusing on yes/no decision values,[7] potency, pathways,[9] or complex mechanistic analyses. This clearly distinguish genomic test principles from all other more traditional approaches and consequently holds tremendous promise for advanced testing of cosmetic ingredients.

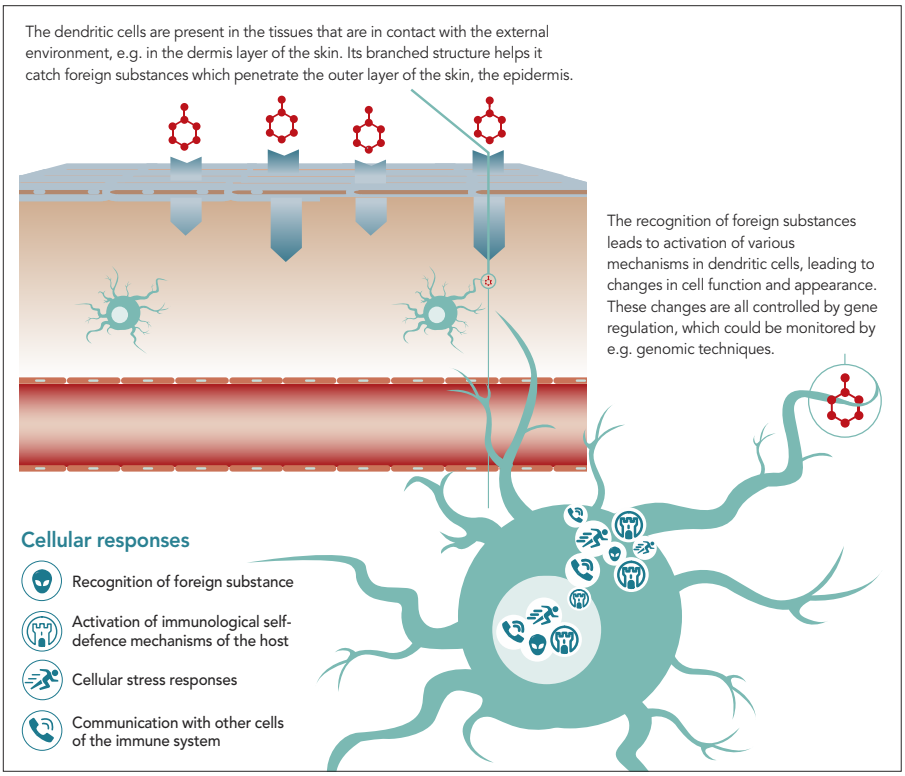


Figure 1: Schematic view of the cellular response to foreign substance in the dendritic cell being an important part of the human immune system.

Animal ban

Even if the 'three R principle' (Reduction, Refinement and Replacement of animal experiments) is more of an ethical code by which scientists are encouraged to perform experiments, rather than a legislation, it permeates the entire industry. The three R principle was outlined in Directive 201/63/EU and is effective since January 2013. Replacement refers to methods that replace the use of animals in all experiments in which reasonable alternatives are available. Reduction refers to methods that minimise the number of animals sacrificed and refinement refers to improvement of scientific procedures that minimise the potential pain, suffering or distress caused to the animals. Legislation such as the 7th amendment to the Cosmetic Directive, adopted in 2003, made research towards development of animal-free test methods really start in order to ensure the availability of test methods fulfilling the regulatory demands towards safe cosmetic products when a final ban would come into place. This became a reality in March 2013 in Europe, preceded by a testing ban on finished products in 2004, and on testing of ingredients and marketing thereof in 2009.

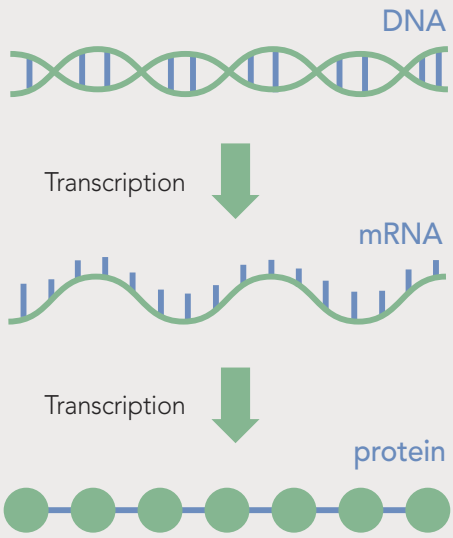
Since 2013, animal testing has also been banned in the cosmetics industry in several other countries outside of Europe, such as India, Israel, certain states in Brazil and New Zealand, and this trend is rapidly spreading

Genomics explained

What is the relationship between DNA and mRNA and proteins?
Life on Earth is based upon the organized activity of proteins. The chemical structure of these proteins is described by the DNA. In multicellular organisms, the DNA is stored in the cell nucleus, whereas the production of proteins takes place outside. The information is transferred from the nucleus to the protein-building machinery in the form of mRNA, messenger RNA. It may be compared to a blueprint which in this case controls the design of the protein structure.

What is 'omics'?
The study of the genome is called genomics. An entire family of related –omics areas has been established: transcriptomics (mRNA), proteomics (proteins), metabolomics (products of the metabolism) et cetera.

What is a dendritic cell?
The dendritic cell is a type of white blood cell (leukocyte). It serves as a sentinel which catches antigens, for example foreign substances that have entered the body. Once activated, it will migrate to local lymph nodes where it alerts and interacts with other white blood cells of the immune system, primarily T cells.



What is a T cell?
The human immune system – protecting us against microbes and foreign substances – consists of a basic, universal defence which we are born with, and a customised defence against things that we encounter during our lifespan. The T cells carry our 'immunological memories' and make it possible to develop immunity against diseases we have previously encountered.

What is sensitisation?
Chemical hypersensitivity (also called chemical allergy) is a disease state induced by the human immune system in response to chemical sensitisers and is initiated by a process termed sensitisation. Sensitisers activate dendritic cells and initiate an immune reaction. Sensitisation occurs when the T cells learn to recognise a specific sensitiser. Following subsequent exposure, the primed T cells – which have now established an immunological memory – react rapidly to induce a state of inflammation. This in turn leads to the disease-associated symptoms, such as itching, blistering and tissue damage.

across the globe. The most recent action came in June this year when President Obama encouragingly signed a law that places stronger regulations on chemicals present in nearly every product in American use.

Alternative methods

As a direct consequence of the abovementioned development, alternative methods for assessment of skin sensitisation have become a major focus of most national research efforts in both industry and academia.

The animal method to be replaced for testing of skin sensitisation is the Local Lymph Node Assay.[10] The LLNA is a murine model, which was developed to evaluate the skin sensitisation potential of chemicals and to serve as an alternative approach to the, at that time, traditional guinea pig methods and to provide important animal welfare benefits. The LLNA was at that time a successful example of 3Rs. The LLNA has served as a gold standard in the field of skin sensitisation for many years and a substantial number of scientific reports on its performance have been delivered, outlining an overall accuracy of 70%-75%.

When designing a predictive test for humans it is important to realise that the only true predictive model organism for humans is the human itself or cell systems mimicking the human system. There are >200 different human cell types, so which one to select? The principle behind a predictive test must be to design an *in vitro* system that mimics the reaction in the human situation, as closely as possible. In skin sensitisation, one should aim to mimic the human immune system, since this would generate a read-out as close as possible to a human sensitisation reaction. One central cell in the immune system, orchestrating a variety of immune processes, such as different T cell responses, is the dendritic cell (Fig 1).

Consequently, a logical choice is to base a test system on dendritic cells, in particular one that resembles the *in vivo* counterparts as closely as possible. The cellular and transcriptional activity of the chosen cell line in response to stimuli should parallel the human immune reaction, as well as form the basis of a test that can be standardised and quality controlled. Furthermore, the read-out of a reliable alternative method should be based on as many parameters as possible, since this renders the test a robustness not achieved when focusing on single markers. Provided that the cells utilised for assay development play a role as decision-makers in the immunologic response to foreign substances, such cells should be explored for biomarkers associated with sensitisation.

To date there are three alternative methods for testing skin sensitisation that have been validated and approved by the EURL-ECVAM. These are Direct Peptide Reactivity Assay (DPRA), Keratinosens and Human Cell Line Activation Test (h-CLAT). These tests are based on single marker read-outs such as peptide reactivity, or expression of Keap1-Nrf2 and CD86 and CD54. Single read-out parameter systems contain limited information which might be the underlying reason to the reported accuracy of these methods, rarely exceeding 75%.

DPRA is based on the fact that when initial exposure of a chemical or hapten to the skin occurs, the chemical binds to a protein carrier in a process known as haptenisation. The DPRA mimics this covalent binding of electrophilic chemicals to nucleophilic centres in skin proteins, and by quantifying the reactivity of chemicals towards model synthetic peptides containing cysteine and lysine the exposed chemical can be classified as a sensitiser or a non-sensitiser.[11]

Keratinosens, on the other hand, is based on the fact that the Keap1-Nrf2-ARE pathway have been shown to be a major regulator of cytoprotective responses to oxidative stress or electrophilic compounds and are known to be involved in the cellular processes in skin sensitisation. The assay is based on an immortalised adherent human keratinocyte cell line (HaCaT cell line), transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-

antioxidant/electrophile response element (ARE).[12]

Finally h-CLAT is based on the fact that CD86 and CD54 are surface molecules that are typical markers of dendritic cell activation and play a role in T cell priming, both important steps in a sensitisation reaction. It uses THP-1 cells, a human monocytic leukemia cell line, as the test cell. The cell surface marker expression of CD86 and CD54 is measured by flow cytometry and the relative fluorescence intensity of the surface markers are calculated, leading to a classification of the test substances as sensitisers or non-sensitisers.[13]

A new multiparametric genomic test

GARD (Genomic Allergen Rapid Detection) is the result of many years of research regarding the immune system and its role in allergic reactions at the Department of Immunotechnology at Lund University in Sweden. The department participated in the European Commission 7th framework programme 'Novel Testing Strategies for in vitro Assessment of Allergens; Sens-it-iv' during 2005 – 2010, which later formed the basis for GARDskin.

Since this new test is based on the multiparametric principle harbouring massive amounts of information the test offers an unparalleled way to make hazard predictions. To assure that the complexity of immunological responses is captured, artificial assay simplifications are avoided by implementing a holistic approach for data analysis, using genomics. By using a panel of reference chemicals, including 18 well-known sensitisers, 20 non-sensitisers and vehicle controls, a vast number (200) of differentially regulated transcripts have been identified, related to whether the cells were exposed to a sensitiser or a non-sensitiser. The 200 identified transcripts are known to be involved in immunological relevant pathways that regulate recognition of foreign substances. Thus, these biomarkers are highly relevant predictors of different sensitisers and are used to analyse the outcome when the test cells have been exposed to a foreign chemical substance. As a consequence of the massive amounts of information generated by GARD it also covers several of the key events in the adverse outcome pathway leading to sensitisation.

The new assay has been scientifically validated in-house with, (i) chemicals blindly provided by the cosmetic industry;14 (ii) in collaborative project with industry and (iii) by analysing a large set of blinded chemicals provided by Cosmetics Europe (manuscript in preparation). The accuracy of the test is repeatedly around 90%.

The assay has been commercially available from SenzaGen since 2013 and is currently being evaluated by ECVAM and has been included in the OECD Test Guideline Programme (TGP) work plan with the TGP No 4.106, to gain internationally recognised validity.

Principles of the protocol

Instead of using an animal model for chemical safety assessments, SenzaGen's *in vitro* assay is based on a proprietary human cell line mimicking features of immunoregulatory cells.

In order to decide the appropriate concentration of each test substance used for final stimulation of the cell line, the cells are exposed to the chemical of interest in different concentrations and incubated for 24 hours. Viability of the cells is measured by flow cytometry analysis of propidium iodide stained cells. The particular concentration of a chemical inducing a relative viability of 90% (RV90) is selected for the final stimulation.

The substance to be tested is then used to expose SenzaGen´s proprietary cell line in a concentration determined by the RV90 value, as described. Benchmark chemicals with known sensitising properties are used to calibrate the result, and all stimulations

Figure 2: SenzaGen's GARD cell technology in 5 steps.

are performed in triplicate samples.

The challenge of replacing animal studies by *in vitro* assays requires systems reflecting the complexity of sensitisation. Consequently, GARD is based on a multiplex genomic technology, quantifying the expression levels of identified biomarkers and using multivariate analysis to distinguish sensitisers from non-sensitisers. Consequently, the RNA (the product of gene expression) from the stimulated cells is harvested for further analysis. SenzaGen has implemented the Nanostring platform to measure gene expression of the selected set of biomarkers. In the case of the new test, it is based on 200 genetic biomarkers, giving a robust information rich read-out. The Nanostring method relies on colour-coded probes, specifically designed for the new multiparametric genomic test, which are hybridised to the target RNA and then quantified. The gene expression data from the 200 genes are deciphered by an in-house developed bioinformatic model, resulting in the classification of sensitisers and non-sensitisers.

The underlying cell technology is based on five steps, as shown in Figure 2. The steps are:

Step 1: A human cell line mimicking the human immune system is used as target for exposure of substances to be tested.

Step 2: The cells are exposed to the substance to be tested.

Step 3: Their genomic product (transcripts) are isolated for downstream quantification.

Step4: The gene transcripts are quantified using the multiplex Nanostring technology.

Step 5: The readout is processed using advanced computer algorithms to assess a chemical's ability to induce an allergic reaction.

These steps have today been turned into a simple, industrialised, and robust process that has already been implemented in industrial and regulatory applications. PC

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