The LUSH Black Box Prize and the skin sensitisation adverse outcome pathway

Dr Gill Langley, a science judge on the Lush Prize judging panel, asks whether the Prize judges could be making the first Black Box Award in 2015 for work on skin sensitisation.

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Introduction

The Lush Black Box Prize offers, in any one year, the full £250,000 Lush prize fund for a key breakthrough in human toxicity pathways research using 21st-century techniques. It's called the Black Box Prize because 20th-century product safety testing, relying on animal tests, explains little about whether and how chemicals cause adverse health effects in humans: it treats how chemicals cause toxic effects as a 'black box'. The Lush Prize wants to 'open the box' by aiding the development of advanced human-relevant tests that explain toxic effects, replace the use of animals and improve the science of safety testing.

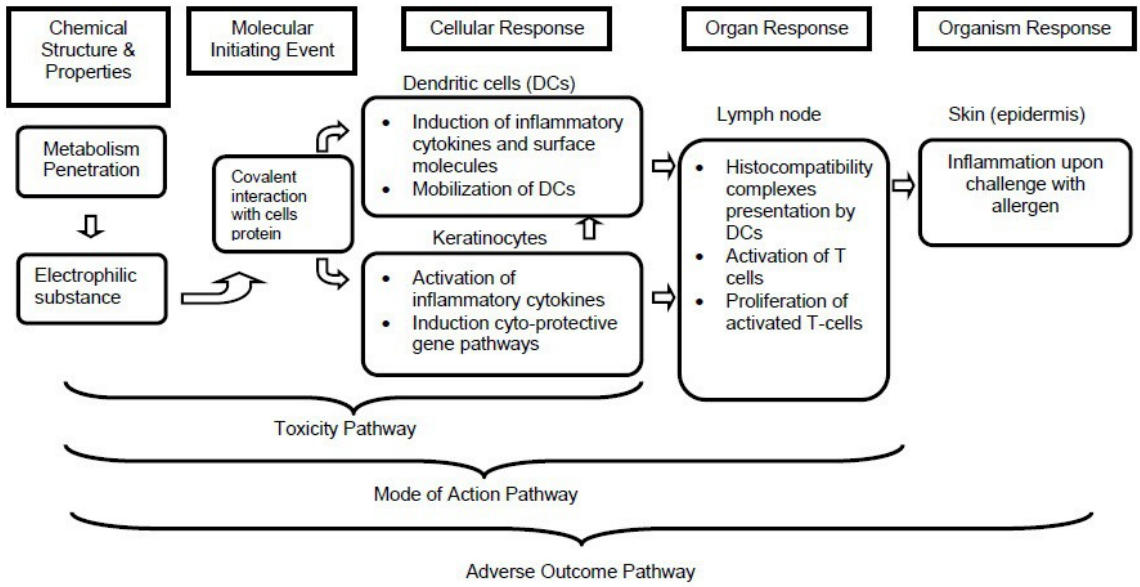
The aim of the Black Box Prize is to stimulate a worldwide research and training focus on human toxicity pathways, with a view to replacing animal tests in toxicology. This would drive forward the culture change in the world of toxicology, which has already started as a result of the U.S. National Research Council's 2007 report [*Committee on Toxicity Testing and Assessment of Environmental Agents, National Research Council (2007). Toxicity Testing in the 21st Century: A Vision and a Strategy*]. See Appendix 1 for an explanation of 21st-century toxicology.

What's a toxicity pathway?

A toxicity pathway is the molecular sequence of events inside the body's cells that follow when a toxic chemical first interacts with the body. Toxicity pathways develop when normal healthy cell pathways are damaged, resulting in changes in gene function, cell signalling or protein production, and injuring the healthy function of the cells. The eventual result is seen as a toxic effect in the whole human, such as when a chemical carcinogen causes a tumour.

Although most toxic effects ultimately involve a complex interplay of different tissues, organs and body systems, toxicity pathway research focuses on the early events at the molecular and cellular levels (see Figure 1, below). The Adverse Outcome Pathway (AOP) concept is a related approach that looks at the entire chain of events from chemical exposure through to effects at the level of the whole organism or groups of individuals (populations).

Figure 1



Flow diagram showing different pathway concepts in skin sensitisation (skin allergy)

Key toxicity pathways are being researched using computational models and human molecular, cellular and tissue studies in the test tube. When pathways are understood, non-animal tests can be developed which identify whether or not chemicals trigger these pathways and eventually lead to toxic effects.

21st-century toxicology offers dramatic advantages over traditional animal testing: speed, human relevance, cost-effectiveness, understanding the causes of toxicity, predicting human variability, testing chemical mixtures, and the replacement of animal testing, which causes suffering to many thousands of animals every year.

Why are toxicity pathways so important?

There are expected to be hundreds of toxicity pathways, variously associated with different tissues and toxic endpoints (e.g. skin, liver, kidney, nervous system) in the body and with different classes of chemicals. A single chemical may activate several toxic pathways, but there is probably a smaller core of cellular events that will provide the key targets for new safety tests.

Modern thinking is that new non-animal tests will be best developed on the basis of pathways.

The process of pathway elucidation, test development, validation and acceptance typically takes many years' work by many researchers and regulatory experts, and roughly comprises:

1. Elucidate the pathways causing a particular toxicity
2. Identify the key cellular steps
3. Devise complementary non-animal tests (chemical, computational and/or *in vitro*) to detect and measure the key steps
4. Conduct multi-laboratory assessments of all the tests and devise a way of interpreting the data
5. Achieve successful validation studies
6. Develop a weight-of-evidence (or integrated testing) strategy that combines the tests in a logical framework allowing decisions to be made about toxicity; or a chemical categories-based assessment (grouping chemicals based on both up-stream chemical and down-stream biological processes)
7. Achieve regulatory acceptance for the tests (e.g. European Commission, OECD).

Research that could win a Black Box Prize

The Lush Prize website says:

"Research is eligible for the Black Box Prize if it fully elucidates and describes a human adverse outcome pathway, with experimental evidence to demonstrate all the necessary key steps from the first interaction of one or more chemical molecules to the full effects at the cellular, tissue or individual level. Research must have led to the development and regulatory acceptance, ideally at OECD level, of one or more non-animal tests that replace some or all existing animal tests related to this pathway. The research should have been completed and published within five years prior to the annual award."
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The skin sensitisation pathway

The toxic endpoint of skin sensitisation (SS) is allergic contact dermatitis. When someone's skin is repeatedly exposed to an allergenic chemical, the following symptoms usually follow: a skin rash that can ooze, drain or crust and become raw, scaled or thickened. Itching, skin redness or inflammation and localised swelling follow, and the skin may blister. If left untreated, the skin may darken and become leathery and cracked. Once someone has become allergic to a substance they will always react to it.

The first fully explained and described toxicity pathway now exists (see Figure 1 and Appendix 2), for skin sensitisation caused by chemicals via a reaction with skin proteins.

There are other pathways of skin sensitisation (SS) caused by other kinds of allergens (e.g. metals) and knowledge of the underlying biology of some of the key steps for these other allergens still remains incomplete.

Work to elucidate the pathways leading to SS has been underway for very many years, and like most research, progress has come gradually. Chemically induced SS involves complex mechanisms with many levels of fine control. Several different kinds of cells interact including the skin and the local lymph nodes of the immune system (see Appendix 3 for diagram and explanation).

The full toxicity pathway and AOP for SS caused *by chemicals via a reaction with skin proteins* are now sufficiently understood, and non-animal tests are under development or are being assessed for their validity (see Appendix 4).

The SS AOP is summarised as eleven steps with four key steps (see Figure 1 and Appendix 2):

- Key step 1 is the molecular interaction of the chemical with skin proteins
- Key step 2 is an inflammatory response and change in cell signalling pathways inside skin cells
- Key step 3 is activation of dendritic cells
- Key step 4 is T-lymphocyte proliferation

It will not be possible to assess all SS with only one non-animal test. Several complementary tests will be needed, which might target any or all of these steps (and other steps identified as important for different kinds of allergens, in the future). The tests will also need to provide a measure of how allergenic substances are, i.e. their potency.

But without knowing the toxic pathways, the relevant non-animal tests could not be developed with any confidence in their reliability.

Significance of the skin sensitisation pathway

Since the Lush Prizes were launched in 2012, the SS pathway caused by chemicals via a reaction with skin proteins is the first to be elucidated. Understanding the first SS pathway is a highly significant achievement, for these reasons:

1. SS isn't a simple 'topical' toxicity endpoint (like skin irritation) – it involves a systemic response because the skin *and* the immune system play a part.
2. 'Opening the black box' for SS sets a vitally important precedent by showing that discovering human toxicity pathways for other, more complex toxic endpoints really is achievable – as envisaged by the US National Research Council in 2007.
3. The achievability of understanding toxicity pathways is shown by the fact that the OECD has set up an international AOP Development Programme.
4. This signals that replacing animal tests for all the other endpoints using toxicity pathways is practical, and in this sense does indicate the beginning of the end for animal tests.
5. At present there are no validated non-animal tests for SS although several are being developed (see Appendix 4). Tests on mice (the Local Lymph Node Assays) and guinea pigs (the Guinea Pig Maximisation Test and the Buehler Test) are the

only accepted methods. Thousands of these animal tests are conducted every year (nearly 1,000 in Britain alone in 2013).

6. SS is a significant human health problem caused not only by cosmetics and fragrances, but also by a wider range of chemicals, metals and drugs. The understanding gained in research into the first SS pathway will help understanding of other SS pathways – and therefore the faster replacement of animal tests.

Should a Black Box Prize be awarded for the SS pathway?

According to the Lush Prize policy (see Research that could win a Black Box Prize, above), the SS adverse outcome pathway (AOP) appears to be eligible for a Black Box Prize,and includes kep steps at the system and organ levels, not just the cellular level (see Figure 1 and Appendix 2).

It is difficult to pinpoint the date of publication of the complete pathway, because so many researchers were involved in a long developmental process. To win the Black Box Prize for 2015 the research should have been published not earlier than 2010.

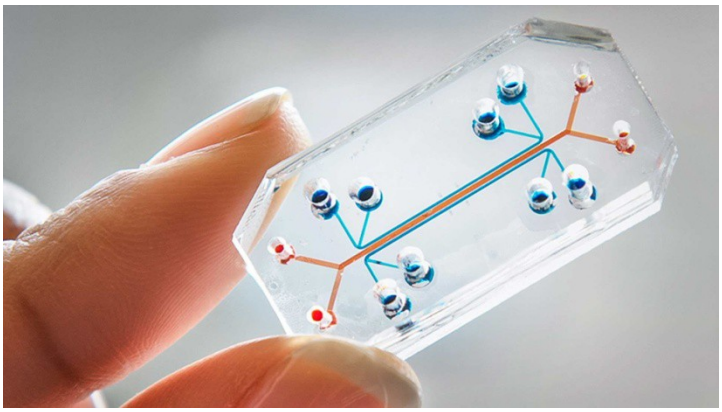
Certainly, the OECD published a report in 2012 [*The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins, Part 1: Scientific Evidence* [www.oecd.org/officialdocuments/displaydocumentpdf/ ?cote=env/jm/mono(2012)10/part1&doclanguage=en] which concluded that the steps in the pathway are sufficiently well understood to devise appropriate non-animal tests for SS. The OECD is the major player in producing test guidelines for chemical testing, agreed internationally.

This could be considered as an official “approval” of the value of the SS pathway. The OECD has also accepted the AOP concept as a framework for human health toxicology within its AOP Development Programme.

Lush Prize welcomes comment and debate around this issue. Please use the contact us link at the bottom of all our web pages.

APPENDIX 1
What is 21st century toxicology?

21st century toxicology is so called because it applies new and emerging techniques and models developed in this century, such as genomics, proteomics and the other 'omics, three-dimensional human tissue models, modern analytical microscopy, mathematical modelling and organs-on-chips. Computational systems are used to integrate and interpret data from human test-tube studies to create understanding of complex biological processes at the level of the whole human.



'Organs-on-chips' can be used to study human cells working in concert with each other as in real life

A transformation in toxicology has been unfolding since the publication in 2007 of the U.S. National Research Council's report which recommended a "21st century paradigm" for safety testing, involving a radical move away from animal tests towards a completely new framework based on understanding toxicity pathways within human cells.

APPENDIX 2

The 2012 OECD Summary of the AOP for skin sensitisation caused by chemicals binding to skin proteins

(Steps 1 – 6 comprise the toxicity pathway contained within cells, and 7 – 11 are part of the AOP with organ-and system-level steps)

Knowledge of the AOP for skin sensitisation elicited by covalent binding of substances to proteins has evolved rapidly over the past decade and may be summarized as:

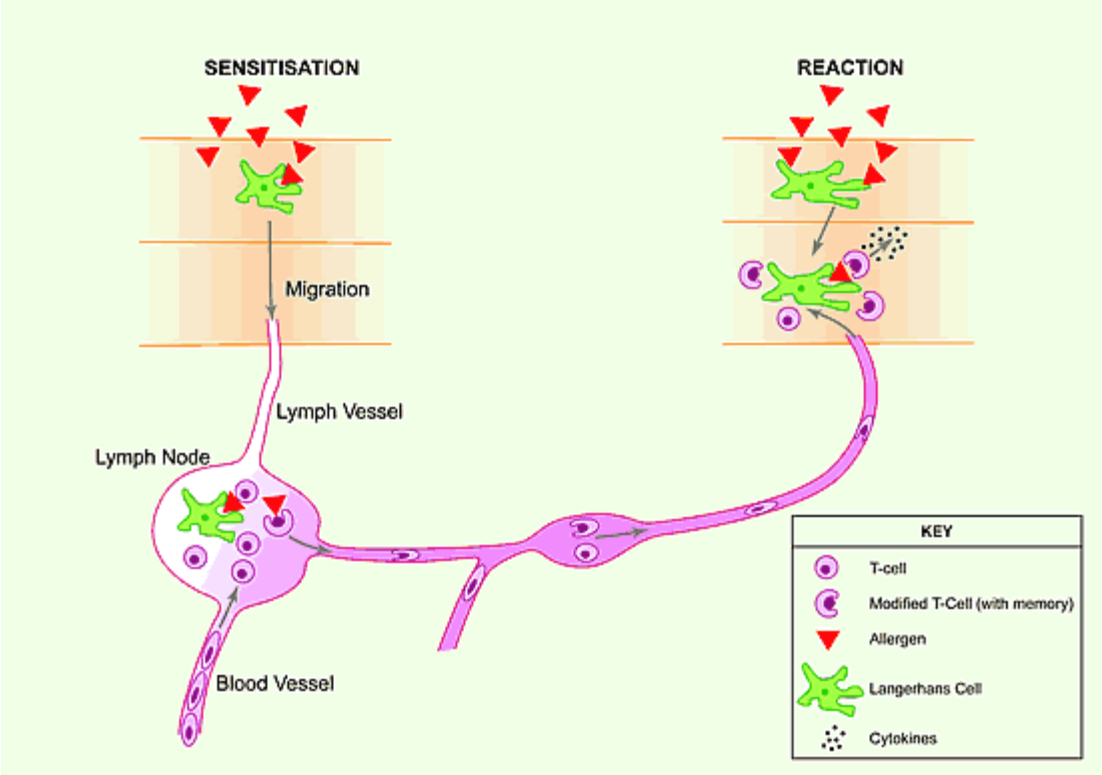
Step 1) The target substance must be bioavailable (i.e. it must penetrate the stratum corneum of the skin).
Step 2) The target substance must be a direct-acting electrophile, be converted from a non-reactive substance (pro-electrophile) to a reactive metabolite via metabolism, or be converted from a nonreactive substance (pre-electrophile) to a reactive derivative via an abiotic process, typically oxidation.
Step 3) The molecular sites of action are targeted nucleophilic sites in proteins (e.g. cysteine and lysine residues) in the epidermis.
Step 4) The molecular initiating event is the covalent perturbation of dermal proteins, which is irreversible (i.e. formation of the hapten-protein complex or complete antigen). *In vivo*, this event is associated with the production of a specific memory T-cell response.
Step 5) Biochemical pathways affected by the definitive electrophile's action on the molecular targets are incompletely known but often include inflammation-related pathways, including the mitogen-activated protein kinase signalling pathway and the oxidative stress response pathway, especially in keratinocytes and dendritic cells.
Step 6) The cellular/tissue-level outcomes are incompletely known but include epidermal responses such as: 1) immune recognition of chemical allergens by keratinocytes, specialized epidermal dendritic cells (i.e. Langerhans cells) and dermal dendritic cell; 2) responses in the form of expression of specific cell surface markers, such as adhesion molecules, chemokines, and cytokines such as IL1β or IL-12p70 are typically taken as evidence of dendritic cell maturation.
Step 7) The organ-level responses include:
a) Dendritic cell migration to the lymph node, where they present major histocompatibility complex (MHC) molecules to naive T-lymphocytes (T-cells), and
b) T-cell differentiation and proliferation as allergen-specific memory T-cells.
Step 8) The target organ(s) are the skin and local lymph nodes; the target cell populations are the immune cells, especially effector T-cells.
Step 9) The key physiological response is acquisition of sensitivity.
Step 10) The key organism response is dermal inflammation upon receiving the substance challenge in the elicitation phase. This response is associated with stimulation of specific memory T-cell produced in the induction phase.
Step 11) The overall effect on mammals is allergic contact dermatitis in humans, or its rodent equivalent contact hypersensitivity.

APPENDIX 3

Brief summary of the skin sensitisation reaction

The process is sequential. First is sensitisation (or induction) when first exposed to a chemical, then elicitation of the skin condition which reacts on repeat exposure to the chemical. The skin is exposed to an allergy-causing chemical (or metabolises a chemical to a reactive form) and dendritic cells in the skin are activated. These take the chemical and present it to the T-lymphocyte cells in the nearest lymph nodes.

These T-cells then multiply so that if the chemical allergen is experienced again by the individual, these T-cells will respond more quickly and more aggressively. The reaction they create by releasing cytokines (which cause inflammation) in the skin leads to allergic contact dermatitis.



APPENDIX 4

Progress with non-animal tests for skin sensitisation

A number of non-animal tests are under consideration for assessing the SS hazards of chemicals, but none has yet been fully approved by regulatory agencies. Some of the tests have been worked on for 10 years, but until the understanding of the SS toxicity pathway/AOP, it was difficult to prove that any or all of them were reliable and relevant in predicting chemical toxicity for humans.

The formal concept of 21st-century pathway-based toxicology has only existed since 2007. Enormous progress has been made since then. For example, most of the evidence indicating that the Nrf2-Keap1-ARE regulatory pathway is a toxicity pathway for SS was accumulated between 2007 and 2009. The Direct Peptide Reactivity Assay (DPRA), a chemical test, was first developed in 2007 and has gained much credibility in recent years.

With understanding of the SS pathways, and with subsequent pathway elucidation for other toxic endpoints, non-animal test methods are progressing faster and will gain the confidence of scientists and regulators more quickly.

Non-animal tests currently under discussion at, and recently approved by, the OECD (considered the ultimate stamp of approval) include:

- The Direct Peptide Reactivity Assay (DPRA) is now an official OECD test guideine the OECD (since 2012) as a new OECD test guideline. It is based on key steps 3 and 4 of the skin sensitisation AOP and quantifies the reactivity of chemicals to synthetic peptides.
- The KeratinoSens (or *in vitro* ARE-Nrf2 Luciferase) test is based on the second key step in the AOP. The OECD has drafted a new test guideline for this test.
- There is also a draft proposal for a new test guideline on *in vitro* skin sensitisation testing using the human Cell Line Activation Test (h-CLAT). The test detects the third key step (dendritic cell activation) of the skin sensitisation AOP.

Yet other tests are under development as this is an active research field, thanks to the stimulus provided by acceptance of the 21st-century toxicology concept.