# Chemical Respiratory Allergy: Opportunities for Hazard Identification and Characterisation

## The Report and Recommendations of ECVAM Workshop 60[a]

**Ian Kimber,[1] Raymond Agius,[2] David A. Basketter,[3] Emanuela Corsini,[4] Paul Cullinan,[5] Rebecca J. Dearman,[1] Elena Gimenez-Arnau,[6] Leona Greenwell,[3] Thomas Hartung,[7] Frieke Kuper,[8] Piero Maestrelli,[9] Erwin Roggen[10] and Costanza Rovida[7]**

[1]Syngenta Central Toxicology Laboratory, Macclesfield, UK; [2]Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK; [3]Safety and Environmental Assurance Centre, Unilever Research, Bedfordshire, UK; [4]Laboratory of Toxicology, Department of Pharmacological Sciences, University of Milan, Milan, Italy; [5]National Heart and Lung Institute, Imperial College, London, UK; [6]Laboratoire de Dermatochimie, University Louis Pasteur, Strasbourg, France; [7]ECVAM, IHCP, European Commission Joint Research Centre, Ispra, Italy; [8]TNO Quality of Life, Zeist, The Netherlands; [9]Department of Occupational and Environmental Health, University of Padova, Padova, Italy; [10]Novozymes A/S, Bagsvaerd, Denmark

## Preface

This is the 60th Report of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). The main goal of ECVAM, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods that have scientific relevance and that reduce, refine or replace the use of laboratory animals. One of the first measures taken by ECVAM was the implementation of procedures that would enable it to become better informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. The decision was taken that this would be best achieved by the organisation of ECVAM Workshops each addressing a specific topic, and at which selected groups of independent international experts would review the status of various types of *in vitro* tests and their potential application, and make recommendations about the best way forward.

A workshop on *Chemical Respiratory Allergy*, chaired by Ian Kimber, was held at ECVAM from 11–13 October 2006.

Chemical respiratory allergy, typically associated with rhinitis and/or asthma, is considered to be an adverse health effect of high concern. For example, under the EU Registration, Evaluation and Authorisation of Chemicals (REACH) system, respiratory sensitisers are included among substances of higher risk, together with CMRs (chemicals that are Carcinogenic, Mutagenic or toxic for Reproduction), and are regulated under Annex 1 of *Directive 67/548/EEC*, which contains a list of dangerous substances, including respiratory sensitisers. Although some animal methods (such as measurements of IgE antibody responses in mice, or specific pulmonary reactions in guinea-pigs) are mentioned in this Directive, these methods have not been validated, nor are they widely accepted by the scientific community.

At present, chemical respiratory sensitisation hazard is assigned to chemicals on the basis of epidemiological evidence or, in the case of diisocyanates, as a default classification. Therefore, there is clearly a need to develop new and improved methods for hazard identification and characterisation, and it was the objective of this workshop to explore what opportunities might now be available.

## Introduction

Chemical respiratory allergy, characterised typically by rhinitis and asthma, is an important occu-

pational health problem associated with considerable morbidity and related economic and social costs (1, 2).

Compared with those implicated as skin sensitisers, fewer chemicals are known to cause sensitisation of the respiratory tract. These include: diisocyanates (such as toluene diisocyanate [TDI]), acid anhydrides (such as trimellitic anhydride [TMA]), some platinum salts, certain reactive dyes, plicatic acid and chloramine-T (1, 2).

Chemical respiratory allergy poses significant challenges for the toxicologist, not least of which is the fact that there are, as yet, no validated or widely accepted methods available for the identification and characterisation of chemicals that have the potential to cause allergic sensitisation of the respiratory tract. This is attributable, at least in part, to the fact that there is continuing uncertainty about the exact nature of the immunological mechanisms through which respiratory sensitisation to chemicals can be acquired, and pulmonary reactions elicited. The purpose of this report is to briefly review chemical respiratory allergy, with respect to what is known of clinical characteristics, immunological mechanisms, approaches to safety assessment, and relationships that may exist between physicochemical properties and sensitising activity. Against this background, opportunities for the design and development of alternative approaches to hazard characterisation are considered. Finally, key recommendations are made for actions required to sustain continued progress in this area.

## Definitions and Scope

Understanding of respiratory allergy is frequently obscured by the use of several words and phrases to describe the same or similar phenomena. Therefore, but only for the purposes of this report, the following definitions have been used to provide some structure and scope to the deliberations of the workshop participants.

*Allergy* is used to describe *the adverse health effects that may result from the stimulation of a specific immune response*. It characteristically develops in two phases, and to reflect this, the following working definition of *allergic sensitisation* has been adopted: *heightened immunological responsiveness that may result in the elicitation of an adverse reaction following subsequent encounter with the inducing allergen*.

On this basis, *chemical allergy* has been defined as *the adverse health effects that may result from the stimulation of a specific immune response by exposure to a chemical*. Consistent with the available clinical and epidemiological information, a *chemical respiratory allergen* is defined as *a chemical that causes respiratory hypersensitivity, characterised typically by rhinitis and/or asthma*.

It will be apparent that the focus of this report is firmly on a consideration of those chemicals that cause allergic respiratory hypersensitivity (by definition, via a mechanism or mechanisms that involve and require specific immunological responsiveness). It is appreciated that there are chemicals that have been implicated in causing or exacerbating asthma via non-immunological mechanisms (asthmagens). Although such chemicals are not embraced within the remit of this report, they are briefly considered.

Finally, it is worth emphasising that the focus of our attention is exclusively on low molecular weight chemicals. Respiratory allergy provoked by other allergens, primarily by protein allergens, is not considered.

## Clinical Aspects of Chemical Respiratory Allergy

The label of 'occupational asthma' is generally reserved for a disease that arises *de novo* from exposure to an agent encountered at work. It is distinguished from 'work-exacerbated' asthma, where pre-existing disease is provoked or exacerbated by workplace exposures. These two types of work-related asthma carry similar and serious social and economic consequences for the affected individuals (3), but most legislatures consider it important that they are treated separately. The focus of this report is on the former situation.

In most cases, occupational asthma is the result of a specific immediate-type hypersensitivity to a sensitising agent encountered in the workplace, although there may be important accessory mechanisms (see below). This type of occupational asthma is distinguished from 'irritant-induced asthma' resulting from single, high intensity exposures ('gassings') to irritant fumes or gases.

Rhinitis may develop as the result of one or more exposures in the workplace, in which case it is also termed 'occupational'. The mechanisms of occupational rhinitis have been less closely studied, but are presumed to be similar to those that give rise to asthma.

Save for their temporal pattern, the symptoms of occupational asthma are no different to those of asthma that is unrelated to the workplace. Typically, they develop after a 'latent' period of at least several months following the onset of a new exposure at work. Wheezing, chest tightness, coughing and breathlessness are common, and in most instances, in the early cases, these symptoms are associated with time spent at work, with improvement away from work. As the disease develops, this pattern may become more difficult to discern, especially (as is common) where there is associated non-specific bronchial hyperreactivity, in which case the symptoms are also provoked by irritant exposures or exercise outside the workplace.

Occupational rhinitis has a similar time pattern; common symptoms include sneezing and a blocked or runny nose.

Continuing exposure to the initiating agent usually results in a worsening of the symptoms, and may lead to a poorer prognosis. Because the mechanism is usually one of hypersensitivity, symptoms are provoked by increasingly lower exposure concentrations, generally making both respiratory protection and common pharmaceutical treatments ineffective. Patients with occupational asthma are more frequently admitted to hospital than are other workers (4). However, deaths from the disease are very rare.

The avoidance of exposure to the initiating agent usually results in an improvement in the symptoms, but complete remission occurs in only about a third of all cases (5). A long duration of symptomatic exposure, and a higher age at diagnosis, are believed to be poor prognostic factors, although it has been difficult to disentangle the separate effects of these. The severity of disease at the time of diagnosis may also be important in determining the prognosis. 'Avoidance' of exposure frequently means a change in job or occupation, often with attendant unemployment and loss of income. A recent study from the UK suggests that the lifetime costs of registered cases of occupational asthma in that country are between £72 and £100 million. However, since recognised cases are believed to represent only two-thirds of the true incidence, the real costs could be as high as £133.5 million. Most of these costs are borne by the State (6).

## Aetiological agents

Over 350 agents have been reported to cause occupational asthma; in most instances, there is an overlap with the causes of occupational rhinitis. Lists of these agents are available in textbooks (1) and on websites (7). Although low molecular mass causes are fewer in number than high molecular mass agents in any list of occupational respiratory sensitisers, they still represent an important subset of aetiological agents, numbering between 40 and 60 separate chemical entities. Excluding reports of single or few cases, the most important chemicals that cause occupational asthma include: acid anhydrides, diisocyanates, plicatic acid (from Western Red Cedar), colophony fume, metals such as complex, halogenated platinum salts and persulphate salts, and some acrylates.

## Epidemiology

Occupational asthma is one of the most common occupational lung diseases in industrialised countries (8, 9). Estimates of the proportion of new or recurrent adult asthma that is attributable to occupation, range from 9 to 15%, the higher figure being derived from studies judged to be of higher quality (10, 11). Occupational rhinitis is up to three times more frequent than occupational asthma, and these two conditions frequently occur together — between 76% and 92% of patients with occupational asthma also have rhinitis (12).

The prevalence of work-related asthma derived from cross-sectional studies of working populations exposed to chemicals, ranges from 4 to 54%, but in most cases, the estimates are below 10% (median 8.5%; 13). Differences in the characteristics of chemical agents, in levels of exposure, and in study definitions of asthma, may explain the wide variation in these figures. In addition, the true incidence of occupational asthma obtained in a cross-sectional survey may be underestimated, since employees with troublesome symptoms are likely to leave the workplace at an increased rate.

## Risk factors

Intensity of exposure to a respiratory sensitiser is the most important determinant of occupational asthma. Studies during the past decade have shown dose–response relationships for several agents, including flour, fungal α-amylase, laboratory animal proteins, detergent enzymes, Western Red Cedar, colophony, complex platinum salts, and acid anhydrides (13). However, there is still a lack of information with regard to the existence of "no-effect" levels and whether peak exposures are of greater relevance than cumulative doses.

Various other risk factors for occupational asthma have been studied. Unlike occupational asthma, which arises from sensitisation to high molecular mass agents, atopy is rarely a risk factor for asthma induced by chemical agents; the main exception is disease caused by acid anhydrides (14). Other factors, such as gender, pre-existing rhinoconjunctivitis, and non-specific bronchial hyperreactivity, appear to play little part in asthma due to exposure to chemicals.

Differences in major histocompatibility complex (MHC) class II molecules may affect their relative ability to bind peptides, and thereby the nature of T-cell recognition. Information from workforce studies indicates that MHC class II proteins may be important factors in individual responses to occupational agents, including acid anhydrides, diisocyanates, Western Red Cedar, and complex platinum salts (13). Other studies of genetic polymorphisms suggest that antioxidant enzyme systems, including those related to glutathione S-transferase (GST) and *N*-acetyltransferase, are associated with diisocyanate-induced asthma (13, 15). Genetic associations are not, however, sufficiently strong for use in the identification of sus-

ceptible individuals. Nonetheless, the observation that MHC class II molecules contribute to susceptibility to chemically-induced asthma, provides further evidence of an immune-mediated pathogenesis.

## Pathogenesis

Clinical, functional, and pathological alterations in occupational asthma induced by chemicals are similar to those found in asthma that is unrelated to work. They include an excessive reaction to broncho-constricting stimuli — the functional hallmark of both occupational and non-occupational asthma. The pathogenesis of this hyperresponsiveness, which is generally long lasting and poorly reversible, remains unknown. By contrast, the transient increase in airway responsiveness observed in exacerbations of occupational asthma, seems to be associated with an acute inflammatory reaction in the airways, leading to luminal eosinophilia. The histopathological features of occupational asthma include airway infiltration of inflammatory cells (particularly eosinophils), activation of lymphocytes, and an increased thickness of subepithelial collagen (16).

The mechanisms by which chemicals induce occupational asthma are believed to be mainly related to a specific immune response. This does not necessarily imply an IgE-associated (or IgE-mediated) immunity, but may also involve cell-mediated and mixed reactions. Many low molecular mass agents, such as diisocyanates and plicatic acid, cause asthma that has the clinical and pathologic features of atopic disease but is not consistently associated with the production of either specific IgE antibodies or the upregulation of IgE receptors (17). Consequently, it has been suggested that CD8$^+$ cells play a key role in diisocyanate-induced asthma, and this aspect of immune responses associated with chemical respiratory allergy is considered in greater detail in the next section of this report.

Along with lymphocyte activation, asthma induced by low molecular mass agents is associated with an increase in the number of cells producing proinflammatory cytokines. The observation that monocyte chemoattractant protein-1 (MCP-1) is specifically produced by peripheral blood mononuclear cells upon stimulation with diisocyanate–protein conjugates in subjects with diisocyanate asthma, supports the notion of an immunological mechanism in some forms of the disease (18).

## Diagnosis

In the context of appropriate workplace exposure — especially to a chemical agent recognised as able to induce occupational disease — a history of work-related asthmatic symptoms arising after a suitable period of latency is typical of occupational asthma. However, most authorities require further confirmation of the diagnosis. Common functional methods include the serial measurement of peak flow at work and at home, and the measurement of lung function and/or bronchial hyperresponsiveness either side of a working shift. Specific provocation testing under carefully controlled experimental conditions — in hospital — is widely used in specialist centres. In chemically-induced occupational asthma, the identification of specific IgE to hapten–protein conjugates is possible and diagnostically valuable for only a small number of agents. In disease induced by acid anhydrides, the measurement in serum of IgE antibodies to hapten–human serum albumin (HSA) conjugates has a high diagnostic utility, as does the use of skin prick testing with the same conjugate. Prick testing (but not serum IgE measurement) with complex platinum salts is similarly useful. About 30% of employees with occupational asthma from diisocyanates have an identifiable IgE response to hapten–HSA conjugates. This proportion is probably dependent on the interval since last exposure — a positive test is believed to have high diagnostic specificity, but is of low sensitivity.

## Chemical Respiratory Allergy: Immunological Mechanisms

There is no doubt that many aspects of chemical respiratory allergy and occupational asthma are controversial. Recent reviews identify some of the key issues (19–22), and of particular relevance is a recent report that highlights 100 key questions and needs in occupational asthma (23). Issues that frequently stimulate debate are the characteristics of immune responses induced by chemical respiratory allergens, and the nature of the important immunological effector mechanisms. With some respiratory allergens (acid anhydrides and platinum salts, for example) there is strong evidence to support an IgE antibody-dependent mechanism of allergic sensitisation, comparable with the pathophysiology of respiratory allergy to proteins (24, 25). This is often not the case, however. With the diisocyanates, in particular, it has frequently been reported that many subjects showing symptoms lack detectable plasma IgE antibody. In fact, although the numbers are somewhat variable, it is estimated that IgE antibody can be found in less than half (and in some studies in substantially less than half) of clinically confirmed cases of diisocyanate asthma (24, 26–31). Nevertheless, there is a general consensus that although only a minority of patients with diisocyanate asthma have detectable specific IgE, when it is found, IgE antibody is highly diagnostic (20, 32). It is also true that, with the majority of chemical respiratory allergens that have been studied,

there are examples of symptomatic patients with IgE antibody, even in those instances, such as with the diisocyanates, where such patients are in the minority. Therefore, one possibility is that sensitisation of the respiratory tract by chemicals can be achieved via more than one immunological mechanism, including those that are IgE antibody-independent. The other interpretation is that IgE antibody is associated much more closely with chemical respiratory allergy than is currently appreciated, but frequently goes undetected for largely technical or methodological reasons. Thus, for example, it is well established that designing and preparing suitable hapten–protein conjugates for the detection of antibodies raised against chemical allergens, presents important technical hurdles, and the use of inappropriate conjugates may mean that specific antibody will be missed (33–35). Moreover, it is possible that, in some instances, analyses have been conducted at times when induced IgE antibody is no longer present (at least at detectable levels). It has been estimated that following the cessation of workplace exposure, the plasma half-life of detectable IgE antibody specific for the chemical allergen is in the region of 4 months to 2 years (21, 28, 36). Consistent with this is the observation in one investigation that measurements of specific IgE antibody were more likely to be positive, if blood samples were drawn from subjects within 30 days of the last exposure to the chemical allergen (28).

Against this background, it is possible that the correlation between IgE antibody and the manifestation of rhinitis and asthma associated with chemical respiratory allergy, is perhaps closer than some studies have suggested. However, this does not imply that IgE is necessarily a universal mandatory requirement for the development and expression of chemical respiratory allergy. There may be other immunological mechanisms that operate in tandem with, or independent of, IgE antibody. However, even if IgE-independent mechanisms are relevant, there is evidence that immune responses provoked by respiratory sensitising chemicals are commonly of the selective T-helper (Th)2-type, and are therefore comparable, in general terms, at least, with immune responses induced by protein respiratory allergens (37, 38). Certainly, there is available evidence from experimental studies that chemicals known to cause respiratory allergy and occupational asthma in humans elicit selective Th2-type immune responses in mice (39–43).

Although the predominant immune response to chemical respiratory allergens may be of the Th2-type, it is important to acknowledge that other cells may play important support or regulatory roles. For example, studies reported by Herrick *et al.* (42) revealed that the respiratory inflammatory responses provoked in mice by an inhaled diisocyanate (hexamethylene diisocyanate [HDI]) are mediated by CD4$^+$ Th2 cells, but that CD8$^+$ T-lymphocytes are also induced, and it is conceivable that these provide counter-regulatory activity. There is also evidence in humans that CD8$^+$ T cells, and in addition, interferon γ (IFN-γ)-producing γ/δ receptor T-lymphocytes, are induced in response to chemical respiratory allergens, and may play (as yet undefined) roles in the development of occupational asthma (44, 45). The speculation is that, in common with other forms of allergic disease, immune responses to sensitising agents in healthy and allergic subjects are finely balanced between allergen-specific effector cells and regulatory cells (46).

Finally, in the context of this report, it is relevant to consider the position of chemicals that have the potential to cause allergic sensitisation of the respiratory tract within the broader family of chemical allergens. Many hundreds of chemicals have been recognised as potential skin sensitisers, with the ability to cause allergic contact dermatitis. Confirmed chemical respiratory allergens are far fewer in number. Also, while they are known to cause sensitisation of the respiratory tract, typically associated with rhinitis and/or asthma, they are only rarely, and in some instances never, associated with allergic contact dermatitis. The interesting question, which is of some importance with respect to predictive testing and risk assessment, is why some chemicals cause allergic contact dermatitis, while others preferentially result in allergy of the respiratory tract. The answer appears to reside in the quality of immune response that chemical allergens preferentially elicit. As summarised above, and described in more detail elsewhere, chemical respiratory allergens are frequently associated with selective Th2-type immune responses, whereas skin sensitising chemicals usually induce preferential Th1-type responses (37–43, 47, 48). While this inevitably represents something of an oversimplification, in general terms it holds true.

What is not so clear is why different classes of chemical allergen provoke discrete qualities of immune response. This question has received some attention, but there is as yet no clear answer to it. Nevertheless, there are some clues that may be of relevance. For example, it has been found that contact and respiratory allergens may, under some circumstances, display selectivity of association with either cell-associated or soluble proteins (49). Also, it has been proposed that the tempo of accumulation of activated dendritic cells (DCs) in regional lymph nodes may differ between contact and respiratory allergens, and that this, in turn, impacts on the quality of immune response that will develop (50). Intriguing as these observations are, it must be emphasised that the investigations conducted to date have employed only a very small number of chemicals, and it would be premature to conclude that the differences observed are indicative of a more general paradigm.

# Current and Previous Approaches to Hazard Identification

In contrast to the situation with regard to chemicals which cause skin allergy, no well-validated or widely-accepted test methods for the prospective identification of chemicals with the potential to cause respiratory allergy are currently available (51, 52). However, despite the lack of formal validation, there has been considerable investment over the last few decades in the development of various approaches to hazard characterisation. Such methods can be divided broadly into those that rely on monitoring the events which occur during the elicitation phase of the allergic reaction, and those that rely on the measurement of changes in the adaptive immune response during the induction phase. Relatively few chemicals have been tested with many of these methods, the focus having been primarily on acid anhydrides and the diisocyanates. The known respiratory chemical allergen, TMA, has commonly been used as a positive control, in comparison with the reference contact allergen, 2,4-dinitrochlorobenzene (DNCB), a compound that apparently lacks the potential to cause respiratory allergy. One very important consideration is that of the route used for priming. Although inhalation exposure would appear to be the most relevant route for the development of respiratory allergy, this has often proved rather ineffective in experimental studies (53–55). In contrast, dermal (topical or subcutaneous) exposure to chemical respiratory allergens was very effective for the sensitisation of the respiratory tract in guinea-pigs, rats and mice (56–60). There are reports which detail significant occupational exposure to chemical respiratory allergens via the skin, such as that found in automobile repair shop workers exposed to diisocyanates, despite the use of protective clothing (61). Furthermore, there are reasons to suppose, and some (largely anecdotal) evidence to suggest, that dermal contact could cause respiratory sensitisation to some chemical respiratory allergens, particularly when there is acute exposure to high concentrations resulting from accidental spillages or splashes (62, 63). Both inhalation exposure, and more commonly topical exposure, have been used as the route of sensitisation in various experimental systems which are currently being explored.

As described above, elicitation tests employ both the skin and the respiratory tract as routes for priming (induction); challenges are most commonly by inhalation (whole body or nose/head only), or by intranasal and intratracheal applications (Table 1). In contrast to application to the skin, the sites where the allergen is encountered in the respiratory tract can vary, and depend on the physicochemical characteristics of the allergen, allergen concentration, and the manner of application (inhalation, intranasal or intratracheal). For example, highly reactive chemicals, such as TDI, may not reach the lower airways in sufficient quantities (89). The experimental animals of choice for these studies are generally the Brown Norway (BN) rat or the guinea-pig. The naïve BN rat usually has some background pulmonary inflammation (90), which hampers the investigation of allergic inflammation at the alveolar level (74). The rat is also somewhat less sensitive than the guinea-pig, with higher levels of agonists required to induce the same level of bronchoconstriction. On the other hand, the guinea-pig is known to respond vigorously to inhaled irritants, by developing an asthma-like bronchial spasm, and their anaphylactic responses usually involve IgG1 rather than IgE antibodies (91).

Respiratory allergy is characterised by airway obstruction, non-specific hyperreactivity and/or inflammation. Airway obstruction is assessed by plethysmography, by using a number of parameters, such as changes in respiratory rate or breathing pattern. For example, apnoeic breathing patterns indicative of immediate-onset specific airway reactivity were observed in TMA-sensitised BN rats following challenge (92). There is some debate as to whether the parameter 'enhanced pause' (Penh) in unrestrained plethysmography is useful, because it may not directly relate to airway resistance (93). Non-specific hyperreactivity is commonly investigated by challenge *in vivo* or *in vitro* with cholinergic compounds (94, 95). Inflammation is assessed by histopathological examination of the respiratory tract and by measurement of biochemical and cellular constituents in bronchoalveolar lavage (BAL; 64). Inflammation associated with (sub)acute asthma is most often characterised by eosinophil infiltration and goblet cell hyperplasia and hypertrophy. Pulmonary haemorrhage and inflammation resembling allergic alveolitis, are also observed. In addition, allergic laryngitis and rhinitis may be induced by chemical allergens.

The severity and type of respiratory allergy may be directly related to exposure levels during sensitisation and/or challenge. Such dose–response relationships have been found in experimental animals (57, 58) and in humans (96, 97). Irritation of the respiratory tract, induced by the allergen itself, was observed at higher challenge concentrations than those required to elicit an allergic reaction (57, 58), which is consistent with the observation that the exposure concentration necessary to provoke symptoms in allergic individuals may be lower than that which causes irritation reactions (98). However, when using animal models, others have found that challenge concentrations exceeding the irritant threshold concentration are required to elicit functional allergic airway responses (72, 99, 100). It must be noted that there are some dissimilarities between animal models and human respiratory allergic diseases. Late asthmatic airway responses

## Table 1:    Elicitation models for detecting respiratory allergy[1]

| Animals | Sensitisation method | Elicitation method | Parameters evaluated |
|---|---|---|---|
| Guinea-pig | **Inhalation**<br>Single and repeated exposure | **Inhalation**<br>Challenge with hapten or hapten–protein conjugate | Airway responses; histopathology (65–69) |
| Rat | Repeated exposure | Challenge with hapten | Airway responses (70) |
| Mouse | **Intranasal**<br>Repeated exposure | **Intranasal**<br>Repeated (2) challenge | Histopathology; cytokine levels (43) |
| Guinea-pig | **Dermal**<br>Single or repeated intradermal injection | **Inhalation**<br>Challenge with hapten or hapten–protein conjugate | Respiratory symptoms; lung histopathology (54, 66, 67, 71–73) |
| Rat | Repeated topical applications | Single or repeated challenge with hapten | Respiratory symptoms; histopathology (55, 57, 58, 70, 74–76) |
| Guinea-pig | **Dermal + inhalation**<br>Single intradermal injection plus repeated inhalation | **Inhalation**<br>Challenge with hapten or hapten–protein conjugate | Airway responses (66) |
| Mouse | **Dermal**<br>Single or repeated topical application | **Intranasal**<br>Single or repeated challenge with (soluble) hapten | Airway responses; AHR; histopathology (60, 77–81) |
| Guinea-pig | **Dermal**<br>Single or repeated intradermal injection | **Intratracheal**<br>(Repeated) challenge with hapten or hapten–protein conjugate | Respiratory symptoms; plasma extravasation (82–85) |
| Mouse | Single intradermal injection + intratracheal instillation with hapten–protein conjugate | Challenge with hapten–protein conjugate | Pulmonary cellular infiltrate (86) |
| Mouse | Repeated topical applications | Challenge | Airway histopathology (87) |
| Mouse | **Dermal**<br>Repeated topical applications | **Dermal**<br>Topical challenge | Evaluation of immediate (1h) and delayed dermal reactions (88) |

[1]*Adapted from (64)*

are prominent in humans, but less so in animals. Airway hyperreactivity (AHR) is continuously present in many asthmatic individuals, even when they are symptom-free, but is often transient in occupational asthma. AHR is transient in animals. The incidence of allergic laryngitis and allergic alveolitis is low in humans, but particulate allergens can induce these symptoms in animals (101–103). Most animal models focus on (sub)acute, but not chronic, allergic respiratory diseases (55, 75, 84, 104, 105), so experimental data on airway remodelling (a common feature of asthma in humans) are rarely available.

The alternative approach has been to characterise the events which occur in the induction phase of respiratory sensitisation. As described previously, exposure to chemical respiratory allergens is associated with selective Th2-type immune responses, whereas chemical contact allergens preferentially induce Th1-type responses. Markers of polarised Th1 and Th2 cell activation include cytokine expression profiles of allergen-activated lymph node cells (LNCs) and differential production of serum (IgE) antibody. Rats and mice have been the species used in these assays. IgE tests are based on the finding that the topical application of immunogenic concentrations (assessed as a function of induced proliferation in draining lymph nodes) of respiratory allergens, such as TMA, provoked significant increases in the concentration of total serum IgE, whereas equivalent doses of contact allergens, such as DNCB, failed to do so (39, 88,

106–108). The major advantages of this test system are that the same sandwich enzyme-linked immunosorbent assay (ELISA) can be used, regardless of the chemical under test, and the lack of requirement for a chemical–protein conjugate. However, it has been reported that there is considerable inter-animal variation in responsiveness to chemical allergens and in serum IgE levels in control BALB/c strain mice (109). The BN rat offers the advantage that sufficient amounts of serum can be obtained from serial bleeds to permit the performance of kinetic analyses of induced changes in serum IgE concentration on an individual animal basis, thereby allowing discrimination between transient and sustained changes in IgE. Analyses using the BN rat have focused primarily on measuring increases in serum IgE levels elicited by topical treatment with TMA (110–112). Treatment with TMA and phthalic anhydride (PA) generally resulted in significant increases in IgE levels, but exposure to TDI or HDI failed to provoke similar changes (113), or induced only relatively weak increases in serum IgE levels after repeated applications. Therefore, the measurement of changes in total serum IgE may not be sufficiently sensitive for the routine identification of chemical respiratory allergens.

Another marker of polarised Th1 and Th2 activation is cytokine expression profiling or "fingerprinting". Under conditions of exposure of equivalent immunogenicity, draining LNCs excised from BALB/c strain mice treated with DNCB secrete high levels of the Th1-type cytokine, IFN-γ, but only relatively low levels of the Th2-type cytokines, interleukin (IL)-4 and IL-10 (114). The converse Th2 cytokine secretion profile was shown to be provoked by topical exposure to TMA (114). This ability to induce either a Th1 or a Th2 cytokine profile, respectively, has been confirmed with a range of other chemical contact and respiratory allergens, and appears to be species and strain independent (47, 114–120). There are some data that apparently conflict with these observations (121, 122). For example contact allergens such as DNCB and oxazolone, have been reported to stimulate Th2 cytokine release (121). The important point is that the divergent cytokine secretion profiles provoked by different classes of chemical allergen are selective, not absolute. Indeed, it is not unusual to detect the measurable expression of Th2 cytokines following treatment with DNCB, albeit at much lower levels than those stimulated by TMA (114, 116). Recently, it has also been reported that DNCB fails to induce the elevated secretion of IFN-γ (122). In these experiments, however, only very low levels of this, and of other cytokines, (<100pg/ml) were recorded, even following stimulation with the T-cell mitogen, concanavalin A (con A), compared with the ng/ml levels reported previously (122). These data suggest that certain aspects of the treatment

and culture of the cells, or of cytokine analyses, are critical to the outcome of cytokine fingerprinting.

Some of the major protocol variations that exist between laboratories seeking to use cytokine fingerprinting in the context of hazard identification, are as follows: a) the kinetics of the dosing regimen; b) the measurement of cytokine message versus cytokine protein; and c) the use of T-cell mitogens to augment cytokine secretion. The polarised cytokine phenotypes described above take time to mature, and LNCs isolated 3 days, rather than the standard 13 days, after the initiation of exposure display a mixed phenotype, with Th1 and Th2 cytokines expressed following treatment with both chemical contact and respiratory allergens (123, 124). Cytokine mRNA expression, as opposed to protein secretion, has been examined by using the reverse transcription polymerase chain reaction (RT-PCR; 116, 118, 125) or the ribonuclease protection assay (RPA; 119, 122, 124, 126, 127). As expected, exposure to TMA induced increased levels of IL-4 mRNA expression compared with treatment with DNCB. However, the Th1 cytokines, IFN-γ and IL-12, did not always discriminate well between contact and respiratory allergens (116, 118, 125). The RPA method appears to be sufficiently sensitive for detecting consistently preferential Th2 cytokine expression following prolonged treatment with respiratory allergens such TMA and TDI (119, 122, 124, 126, 127). However, RNA isolated from DNCB-activated or 2,4-dinitrofluorobenzene-activated LNCs did not express a selective Th1 cytokine mRNA phenotype, particularly with respect to IFN-γ (119, 122, 124, 126, 127). The reasons for the failure to detect increased mRNA for IFN-γ, despite robust secretion of this cytokine, are presently unclear, although it appears that production of this cytokine by draining LNCs is controlled mainly at the level of secretion, rather than transcription. These data suggest that analysis of cytokine expression by RPA might be inappropriate for the discrimination between contact and respiratory allergens. The other major protocol variable is restimulation of LNCs with polyclonal mitogens, such as con A or anti-CD3 antibody, *in vitro* (118–121). There is some evidence to suggest that the addition of such mitogens may alter/bias the cytokine secretion profile with respect to cytokines other than IL-4. In contrast, with the exception of IL-12 protein which is produced constitutively, quiescent LNCs prepared from untreated controls do not generally produce detectable levels of other cytokines (IFN-γ, IL-4, IL-5, IL-10 and IL-13) in the absence of restimulation with a mitogen. These data suggest that the measurement of cytokine expression in the absence of a mitogen may be more selective for allergen-induced effects (124, 128, 129). Furthermore, although it is necessary to restimulate LNCs with con A in order to detect the expression of IL-4 protein, there is sufficient spon-

taneous production of the other cytokines (IFN-γ, IL-5, IL-10, IL-12 and IL-13) for measurement in the absence of restimulation (124, 128, 129). Under some circumstances, restimulation with mitogen may not completely compromise the development of polarised cytokine phenotypes. However, such cytokine secretion patterns are less polarised than those derived following measurement of cytokine production in the absence of treatment with mitogen, particularly with respect to IFN-γ expression. This suggests that the analysis of cytokine profiles in the absence of restimulation may provide a more robust assessment of sensitising hazard (118, 120).

It is also appropriate to consider how cytokine fingerprinting might be performed, in practice, with a novel test chemical. It is recommended that groups of control animals be exposed concurrently to DNCB and TMA, to provide negative and positive controls for respiratory sensitising potential, respectively. With regard to respiratory allergic hazard characterisation, a chemical is considered to have significant potential for respiratory allergenicity, if similar (or higher) levels of Th2 cytokines are induced to those observed in the concurrent TMA control. Another issue with regard to the application of this method is that of dose selection for test chemicals. It is necessary that concentrations are used that are known to be immunogenic (i.e. which stimulate significant proliferation in the draining lymph node). In practice, robust cytokine secretion is not observed at relatively low levels of proliferation (stimulation indices of <10; Dearman and Kimber, unpublished).

In conclusion — to date, various approaches for the prospective identification of chemical respiratory sensitisers have been explored. Elicitation tests can provide information on the nature and severity of the allergic response throughout the entire respiratory tract, and may provide biomarkers to correlate animal data with human data. In addition, thresholds for challenge can be defined, which are expected to be below thresholds for sensitisation. Tests that measure events occurring in the induction phase of the response, namely, the measurement of total IgE or cytokine fingerprinting, show the greatest promise, particularly with respect to animal welfare, cost effectiveness and ease of application. It is important to note that, in these methods, the emphasis is on hazard identification, and that neither approach has been configured to provide information on the relative potencies of chemical respiratory allergens. Such information is required for the accurate assessment of risk to human health. This is, in part, due to the difficulties of extrapolating between dermal and inhalation exposure, and the paucity of dose–response information, as well as the lack of appropriate benchmarks, due to the current lack of understanding of the relative potencies of known chemical respiratory allergens in man.

## Relationships between Skin and Respiratory Sensitisation and Opportunities for Exploiting Skin Sensitisation Test Methods

Skin sensitising chemicals can readily be identified by using one of a number of predictive *in vivo* tests (130, 131). Most commonly, these are the guinea-pig maximisation test of Magnusson and Kligman (132) and the murine local lymph node assay (LLNA; 133, 134). The accuracy of these methods with respect to the prediction of human hazard is reported to be in the region of 90% (135). However, a question not yet addressed in any systematic evaluation is whether these tests also are positive with chemicals that are able to cause sensitisation of the respiratory tract. Experience to date has revealed that chemicals which are known to be respiratory allergens, can induce sensitisation when applied topically to the skin (136). Retrospective analysis of the database used for the initial validation of the LLNA and of the recently published LLNA database (137), reveals that a variety of chemicals known to be associated with respiratory allergy in humans have been tested in this assay, and that they are universally positive (Table 2). The ability of such chemicals to elicit positive responses in the LLNA is perhaps not unexpected, since these substances, as with chemicals that cause skin sensitisation, are of low molecular weight and are able to bind covalently to protein for the acquisition of immunogenicity.

A non-exhaustive series of results summarised in Table 2 demonstrate that the LLNA is able to identify a range of known chemical respiratory sensitisers. However, it does not demonstrate any specificity, since no results with non-respiratory allergens are shown, nor does the test provide any discrimination between these chemicals and skin sensitisers. The consequence of likely high sensitivity in the absence of specificity, is that this approach could be used only to reveal which chemicals are not respiratory allergens, and can therefore be eliminated from further consideration in that context. That is, the current view is that a chemical testing negative in the LLNA can be regarded as lacking the potential for either skin sensitisation or sensitisation of the respiratory tract.

However, it is necessary to point out that the ability to eliminate chemicals as potential respiratory allergens on the basis of a negative result in the LLNA is predicated upon an assumption that it had been possible to test that chemical at a high enough concentration and in a vehicle appropriate for the assay.

Fewer data on respiratory sensitisers exist for guinea-pig sensitisation tests, but the conclusions that can be reached are essentially the same.

**Table 2:  Standard LLNA results for some common respiratory sensitisers**

| Chemical | LLNA result | Reference |
|---|---|---|
| Hexahydrophthalic anhydride | + | Dearman *et al.*, 2000 (41) |
| Maleic anhydride | + | Dearman *et al.*, 2000 (41) |
| Methyltetrahydrophthalic anhydride | + | Dearman *et al.*, 2000 (41) |
| Phthalic anhydride | + | Dearman *et al.*, 2000 (41) |
| Trimellitic anhydride | + | Dearman *et al.*, 2000 (41) |
| Chloramine T | + | Kimber *et al.*, 1994 (138) |
| Cyanuric chloride | + | Gerberick *et al.*, 2005 (137) |
| Ethylene diamine | + | Gerberick *et al.*, 2005 (137) |
| Glutaraldehyde | + | Gerberick *et al.*, 2005 (137) |
| Diphenylmethane-4-4'-diisocyanate | + | Hilton *et al.*, 1995 (107) |
| Hexamethylene diisocyanate | + | Hilton *et al.*, 1995 (107) |
| Toluene diisocyanate | + | Hilton *et al.*, 1995 (107) |
| Ammonium tetrachloroplatinite II | + | Kimber *et al.*, 1994 (138) |

# Structural Alerts for Respiratory Sensitisation: (Quantitative) Structure–Activity Relationships

As is the case with contact allergy, there is a desire to develop alternative approaches for the identification of chemicals that are able to cause respiratory allergy. Various promising routes are currently being explored, but might not yield results for several years. In parallel with these biological studies, another approach is becoming important, and will no doubt become more important, namely, the study of structure–activity relationships (SARs).

## Biological and chemical bases

The two most important factors, external to the host, for the induction and the elicitation of respiratory sensitisation are the inherent sensitising potential of the chemical (potency) and the exposure concentration (environmental factors). Respiratory sensitisation to a chemical may be a multi-step process with various stages, or may even follow different routes. Typically, a state of sensitisation to the chemical is induced when the antigen interacts with an antigen-presenting cell. However, if the chemical is a hapten (as many low molecular weight asthmagens are), it first needs to interact with a 'carrier', such as albumin, to form a 'complete' antigen. Finally, an allergic reaction is elicited. This happens when a subject sensitive to a given chemical is exposed to, or challenged with, the same chemical or a related chemical. Typically, but not universally nor exclusively, IgE antibodies on the surfaces of mast cells and basophils react with the complete antigen, and a clinical response may then develop. Since chemical reactions are involved throughout these biological processes, which will usually result in the development of immediate hypersensitivity by the patient, it is logical to examine to what extent it is possible to relate the chemical structure of molecules (and hence, by inference, their chemical properties) with their propensities to behave as sensitisers.

There is a well established connection between the ability of chemicals to react with proteins to form covalently linked conjugates and their skin sensitisation potentials, and one of the earliest structure–activity studies, with respect to skin sensitisation, was reported in the 1930s. In that context, it was therefore reasonable to conclude that, if a chemical is capable of reacting with a protein, either directly or after metabolism, then it has the potential to be a contact allergen. The SAR principle states that the toxicological properties of a chemical are dependent on the chemistry of the toxin of interest. SARs are increasingly being seen as a potential aid to the prediction of sensitisation as the toxicological endpoint (139).

## Structural alerts and rule based 'expert' systems

Structural alerts are submolecular chemical 'fragments', 'moieties' or groups, the presence of which is used to trigger concern with respect to a possible adverse effect. They are usually derived on the basis of strong, peer-reviewed, clinical, epidemiologic, and toxicological evidence, which specifically, repeatedly and consistently links the effect in a causal manner to chemical entities with the 'structural alert' in their molecule. An important area with an original application for the identification of skin sensitisers, is the development of new computer-based systems (140). These are the so-called

"expert systems", based on the use of large databases, involving, for example, the structural, physico–chemical, and electronic data for a new compound, and comparing them with the data stored in the memory, in order to derive an opinion on the allergenic status of the molecule. In the expert system field, many parameters can be considered and are described as "structural alerts" and/or "risk indicators". In the skin sensitisation domain, a structural alert consist of total or partial chemical structures known to present allergenic risks. They are mainly defined by analysis of the literature and skin allergy databases, and permit the expert system to detect a risk. One system based on this approach is currently marketed under the name of *DEREK* (Deductive Estimation of Risk from Existing Knowledge). This expert system consists of a control programme that analyses the structures of molecules, and a database consisting of rules in the form of sub-structures known to be associated with allergenic activity (141). Risk indicators are physico–chemical parameters that favour allergenic potential. Each positive parameter is considered as an aggravating factor. Examples are the presence of one or more reactive chemical groups, the number of hydrogen bonds, the number of electron pairs on oxygen or nitrogen, the number of NH or OH groups, and the number of metabolisable groups. One of the main limitations of some of these systems is that they rely on a judgement-based 'estimate' of a score to be assigned to the chemical structural parameter. Karol *et al.* (142) postulated structural alerts and went on to develop quantitative (Q)SARs, as described below.

## (Quantitative) Structure–Activity Relationships

An alternative approach is to search for empirical (Q)SARs, by the application of statistical methods to sets of biological data and structural descriptors. They have the potential of providing inexpensive and rapid predictive tools for the hazard identification of respiratory sensitisers associated with adverse health effects. However, the most important considerations relate to the predictive value and reliability of the techniques. Initial qualitative observations (143) were followed by statistical analyses, which, at first, used relatively small data sets (142, 144). Subsequent work involved more-sophisticated methods and larger data sets of respiratory sensitisers, as well as inactive controls, as in the *CASE* and *MultiCASE* systems (145), which identified biophores (analogous to the structural alerts mentioned above) and a 'categorical' SAR (146).

Others have used the calculation of 'hazards odds ratios' followed by logistic regression modelling (147). Remarkably, in spite of the different mathematical and statistical methods employed, these (Q)SARs can consistently achieve sensitivities and specificities to the order of 90% and 95% or higher. Specificity and sensitivity are, in any case, not absolute, but depend on the 'prior probability' within the 'set' that is being tested. Moreover, in a quantitative (intrinsically non-categorical) test, sensitivity and specificity are a continuum (demonstrated as a curve) — and single values are dependent on the test cut-off, so, to a certain extent, specificity and sensitivity can be traded off against each other, depending on the predictive needs. These studies have been evaluated by using 'leave one out' tests or completely new sets (147). Thus, (Q)SARs can easily be confirmed and corroborated, refuted, or refined, through the expansion of the 'learning set', which is constantly growing.

Depending on the assumptions on the likely 'mix' of chemicals to which they could be applied, negative predictive values of 96% can be derived (147). The (Q)SAR methods can easily handle large numbers of chemicals — for example, nearly 500 in the study by Jarvis *et al.* (147) — with an ease unparalleled by laboratory studies. They can address any and all the clinical outcomes of interest (e.g. asthma), not just those resulting from a specific mechanism (e.g. IgE-mediated allergy). One of the published approaches is unique among the potential predictive methods, in that it provides users with an online web-based interface, through which the 'hazard index' of individual chemicals can be freely tested (147). Thus, besides the 'design transparency' offered by the other respiratory sensitisation (Q)SARs in general, it is unique among the potentially predictive techniques, in that it also gives external researchers or practitioners the opportunity to externally validate, test or apply the method.

In conclusion, the potential value and need for further investigation and application of (Q)SARs in understanding, predicting, and hence preventing, respiratory sensitisation has been recognised (13). The strengths of (Q)SARs include the advantage that they can address actual health outcomes of interest, i.e. adverse health effects associated with sensitisation, rather than only those resulting from defined allergic mechanisms. They can handle large sets of data very quickly, and potentially with very good predictive value. This can be achieved cheaply and without sacrificing animals.

## New Opportunities and Alternative Test Methods: Sens-it-iv and Beyond

The challenge of replacing whole animal studies with *in vitro* assays requires systems which reflect the complexity of allergic sensitisation. The process of sensitisation involves the orchestrated interaction between a variety of cells and mole-

cules, in ways which are tightly regulated in time and space.

First, allergens must gain access to the relevant tissues, where they react with soluble or cellular proteins, either directly or following metabolic activation (bio-activation). Allergen-modified proteins are taken up and processed by DCs. The induction of DC maturation can be monitored by changes in gene expression and phenotype. Allergen-modified peptides are presented by DCs to T-lymphocytes, then, depending on the micro-environment, origin and maturation status of DCs, and the activation status of the T-cells, respiratory or skin sensitisation may result. In addition, there is strong evidence that the epidermal cell (EC)–DC cross-talk which occurs in the skin and the lungs during allergic inflammation, may activate DCs to prime naïve T-lymphocytes (148, 149).

This growing understanding has spurred the development of *in vitro* assays for identifying potential allergens. Together, these models may eventually facilitate the modelling, at least to some extent, of the complexity of the *in vivo* situation. However, none of these attempts has yet resulted in standardised assays suitable for validation and for the replacement of animal testing. Major drawbacks of all the existing *in vitro* assays are: 1) a lack of standardised immunologically and metabolically competent human cell lines for hazard assessment; 2) a lack of assays addressing bioactivation and hapten formation; 3) a lack of culture systems suitable for assessing the sensitising potential of water-insoluble compounds; and 4) a lack of relevant markers for the sensitising potency of different classes of chemicals.

## Assessment of chemical alerts

One of the key molecular events in skin sensitisation is the binding of the chemical to endogenous carrier protein, which makes recognition by the immune system then possible. An understanding of the physico–chemical and structural characteristics of both the chemical and the protein target, resulting in the required covalent bond, would facilitate the development of screening tools for hazard identification, even without the use of cell-based assay systems or animal experiments (as discussed, above).

At present, there are two main types of research activity. One focuses on the chemical, and attempts to elucidate a correlation between sensitising activity and the capacity to bind to proteins. The other approach addresses the amino acid specificity, as well as the protein specificity, of this interaction. These general approaches are briefly discussed, below.

The application of *in silico* chemical alerts for the purposes of risk assessment has been in use for some years (150). It has typically been employed in the areas of environmental exposure and toxicology analysis, alongside major human health endpoints, such as mutagenesis (151, 152) and skin sensitisation (153, 154). The current application of SARs in risk assessment is predominantly in relation to endpoints which have discrete, well-characterised biological mechanisms. However, with increasing understanding of more-complex biological mechanisms (including respiratory sensitisation), opportunities for more-advanced approaches may develop (155).

At present, (Q)SAR models are not suitable for use as stand-alone predictive tools for sensitisation, but the potential is there. It is likely that, in the first instance, (Q)SAR methods will be applied in partnership with other approaches to hazard characterisation.

## Interaction between chemicals and the proteins

In the absence of a target skin protein *per se*, *in vitro* investigations of skin sensitisation have focused on model proteins and peptides. Several authors have identified amino acid residues that are selectively and covalently modified by chemicals of interest, by using HSA as a model protein (156–158). However, it has been shown that not all the modifications which occur are necessarily antigenic. A number of studies suggest that the sensitisation potential of a chemical is probably more closely related to its ability to modify specific residues, rather than a large number of amino acids (158, 159).

The accessibility of a chemical to the specific amino acid residues in the 3-dimensional structure of the native protein, in combination with their localisation relative to B-cell and T-cell epitopes, are, without doubt, important parameters for hapten formation (160). Although still a matter for debate, the micro-environment in which the protein meets the chemical may affect the fingerprint of amino acids modified by the chemical, and thus the sensitising potential. Indeed, the environment may affect the conformation of the protein, such that critical residues are made more or less available for chemical modification. A better understanding of linear and conformational epitopes will contribute significantly to the prediction of critical amino acid residues. The identification of relevant epitopes is a demanding task. However, a number of promising methods have been developed, that will assist in the identification of B-cell and T-cell epitopes (161). Especially in the area of B-cell epitope mapping, there are now available promising *in vitro* and computer-based tools for the localisation of linear and conformational epitopes (162, 163).

The importance of the nature of the protein is still unclear. A study investigating the binding of

cinnamaldehyde to human skin homogenates did not show any specific targeting to any particular protein (164). However, Park and co-workers (49) observed that the strong skin sensitiser, DNCB, preferentially modified cell-associated proteins, whereas TMA, a respiratory allergen, displayed selectivity for soluble proteins. Currently, further research is being performed to determine the molecular basis for this selectivity.

Peptides with sequences analogous to parts of human proteins, and synthetic peptides unrelated to any known protein, are also used to assess chemical reactivity (165–167). Due to the nature of peptides, the impact of the 3-dimensional structure on adduct formation cannot be addressed in such systems. However, peptide-based tests have the advantage that the analytical steps are simpler, which makes possible the development of medium-throughput, or even high-throughput, screening assays.

## Cell-based assays

Hazard and risk assessment for potential skin-sensitisers or lung-sensitisers is currently based on animal testing (as discussed above). At present, no alternative *in vitro* tests have been proposed or developed for assessing the respiratory sensitising potential of chemicals. In contrast, some, as yet unvalidated, methods have been proposed for identifying skin sensitising chemicals, including those based on the production of cytokines by keratinocytes (168–170), the induced maturation of DCs (171–176), and the co-culture of keratinocytes and DCs (177). It is possible that some of these assays may also be suitable for the identification of chemical respiratory allergens.

Recently, the EU has sponsored an integrated project, Sens-it-iv, with the overall objective to produce *in vitro* alternatives for these assays, and to develop them to the prevalidation stage.

### a) DC-based assays

Chemical allergens are haptens that must bind to proteins in order to provoke a specific immune response. The haptenised proteins are taken up and processed in the skin by DCs, such as the Langerhans cells (LCs). Hapten-loaded DCs then migrate to the draining lymph node, where they present the allergen, in the context of MHC class II molecules, to T-lymphocytes. During this migration, the capacity of the DCs for antigen uptake is reduced, while their capacity for T-cell stimulation is increased. In this way, DCs have the ability to induce a primary immune response. A range of signals results in DC maturation. Since LCs are difficult to obtain in sufficient numbers, monocyte-

derived DCs, or DCs derived from CD34+ haematopoietic progenitors, have been used for test development (178). Several authors have reported that the exposure of cultured DCs to contact allergens results in the selective up-regulation of various cell surface markers and genes, including CD40, CD54, CD80, CD83, CD86, and MHC class II (172–176). As an alternative to primary DCs, several cell lines have also been used (including the THP-1, MUTZ-3, U937 cell lines), with apparently similar results (179–182).

However, if surface marker expression proves not to provide a sensitive and reproducible method for the identification of chemical allergens, an alternative approach might be to measure induced changes in cytokine production by DCs (including, for example, cytokines such as: IL-1$\beta$, IL-8, IL-10, IL-12p40, IFN-$\gamma$, tumour necrosis factor [TNF]-$\alpha$, and macrophage inflammatory protein [MIP]-1; 171, 169, 183). Recently, Toebak *et al.* have used primary, monocyte-derived DCs, and found that, in contrast to the up-regulation of CD83 and CD86, which could only be induced by three of seven and five of seven allergens, respectively, IL-8 (CXCL8) production was significantly increased after stimulation with all the allergens tested (184). A combination of IL-8 production and the altered expression of surface markers such as CD54 and CD86, may provide a promising strategy. It remains to be determined, what, if any, impact chemical respiratory allergens have on such systems. In this respect, it has been recently demonstrated, by using primary DCs (176), that the contact allergens, DNCB, oxazolone and nickel sulfate, and the respiratory allergens, TMA and Der p1, have an intrinsic ability to polarise toward type 1 and type 2 cytokines, respectively, irrespective of local factors such as those determined by cutaneous or mucosal epithelial micro-environments. The contact allergens, DNCB and oxazolone, induced type 1 DC polarisation, whereas the respiratory allergens induced type 2 polarisation, while nickel sulfate induced both type 1 (TNF-$\gamma$ and CXCL10) and type 2 (decreased IL-12p70/IL-10 ratio) features. These data suggest that DCs may be of use, not only to discriminate between irritants and allergens, but also in screening for the polarising potentials of chemical sensitisers.

### b) EC-based assays

The respiratory epithelium encompasses a diverse range of functions, including biotransformation, the trapping and elimination of inhaled challenges (the muco–ciliary escalator), and the maintenance and repair of normal epithelium. Cultures of the respiratory epithelium are of growing interest as a possible tool with which to identify chemicals with the potential to cause respiratory allergy. The role

of the respiratory epithelium in the biotransformation and processing of inhaled xenobiotics is well known, and one hypothesis concerning the induction of respiratory sensitisation is that this epithelial processing determines the antigenicity and subsequent allergenicity of a chemical. As the respiratory tract has a diverse epithelium (comprising over 40 different cells types), each region having a discrete histopathology, morphology and functionality, this processing activity is thought to vary throughout the respiratory tree. The implication of this is that both the nature of the chemical and site-specific epithelial activity may impact on potential allergenicity. This diversity also presents a significant challenge, which is likely to require the development of a range of *in vitro* models, in order to encompass the variety of typical interactions across the entire respiratory tree. The development of site-specific *in vitro* epithelial cultures models may, in the future, permit the exposure-related characterisation of epithelial interactions for individual aerosols.

Although epithelial culture models are commonly used to investigate toxicity, biotransformation and tissue modelling endpoints, there has been much less use of these cultures for the investigation of respiratory sensitisation and allergy. Interest in this area has been largely restricted to the investigation of inter-cell interactions and signalling pathways in the elicitation of allergic airway diseases. Only limited published data are available on the use of such systems as screening methods for respiratory allergen hazards. One current limitation in the development of such tools is the availability of cultures demonstrating appropriate organotypic interactions. Several culture models of different airway zones exist (such as, for example, A549 alveolar models, and Calu-3 and BEAS2-B bronchial models; reviewed in 185). However, better characterisation of their responses to sensitisation challenge is required. A major advance in this area has been the development of complex co-culture models, which have been used for investigating both direct and indirect interactions between xenobiotics, ECs, macrophages and DCs (186). In the future, such culture systems may provide biomarkers of immuno-processing interactions. To date, much of this research is focused on the direct chemical–cell and indirect interactions in the alveolar region. Other significant progress in *in vitro* systems has been made in the development of multi-differentiated tracheo–bronchial culture models from both 'normal' and immunocompromised (asthmatic) human airway tissues, which may prove to be useful in the identification of non-immunological asthmagens (187).

Another considerable challenge associated with the development of epithelial cultures as screening tools, is ensuring appropriate chemical exposure *in vitro*. Any inhaled chemical will not only interact with co-inhaled environmental airborne chemicals/particles, but also with the moisture in the airflow of the respiratory tract. These interactions may affect not only the deposition site, but also the properties of the chemical. This is true also of interactions in the fluid lining the lung — a heterogenous population of both hydrophilic and lipophilic components that may play a role in the appropriate processing and presentation of the chemical as an allergen (188, 189). In order to ensure that any epithelial model addresses the appropriate chemical, these pre-epithelial interactions should also be considered. Although the challenges associated with the use of epithelial cultures as predictive tools for respiratory sensitisation are sizeable, the continued investment of effort in this field is vital, and the application of leading-edge technologies can only enhance our understanding and capability.

*c) Macrophage-based assays*

Alveolar macrophages are the predominant immune effector cells resident in the alveolar spaces and conducting airways. They are responsible for the elimination of foreign materials reaching the alveolar spaces. Alveolar macrophages are among the first cells to encounter inhaled compounds, and can produce many different mediators that can have a putative role in asthma. Recently, they have also been found to interact directly with DC processes on the apical alveolar surface *in vitro*, suggesting that there may be direct cross-talk with immune effector cells (186). An excessive inflammatory response might perturb gas exchange. This means that alveolar macrophages must be capable of both enhancing and suppressing inflammatory responses, and must be programmed to implement the effector responses appropriate to the needs of the moment. Overall, the experimental evidence indicates that alveolar macrophages have the potential to inhibit the immune activation and inflammatory cell influx into the lungs caused by the inhalation of respiratory allergens. Among possible anti-asthmatic substances elaborated by alveolar macrophages, there are factors promoting Th1 polarisation, such as IFN-γ, IL-12, IL-18, and nitric oxide (NO), or those with generalised anti-inflammatory activity, such as transforming growth factor (TGF)-β, IL-10, prostaglandin E2 (PGE2), and 15-HETE (190). Lung DCs, but not alveolar macrophages, have been shown by adoptive transfer to be necessary and sufficient for Th2 cell stimulation during ongoing airway inflammation, in an ovalbumin-driven murine asthma model (191).

However, it is important to acknowledge that alveolar macrophages collected from lungs previously exposed to an inflammatory agent develop the capacity to cooperate with T-lymphocytes, inducing their blastogenic response, which indicates that

underlying inflammation greatly enhances the susceptibility of the lung to sensitisation by inhaled antigens. Indeed, it has been demonstrated with diesel exhaust particles, that the induction of a pro-inflammatory response among alveolar macrophages represents an important mechanism through which these cells can mediate the development and exacerbation of allergic sensitisation and inflammation.

Furthermore, Valstar *et al*. have demonstrated that TMA-conjugated serum albumin, dose-dependently stimulated the production of NO and TNF-α by primary rat alveolar macrophages and NR8383 cells, whereas TMA alone (due to the high reactivity and ready hydrolysis in water) was without effect (192, 193).

In conclusion, our knowledge of the regulatory role of alveolar macrophages in allergic asthma requires further elucidation, and the effects on these cells of both chemical allergens and pulmonary irritants should be further investigated.

### Sens-it-iv — exploring innovative approaches

The ultimate goal of the EU Framework Programme 6 Sens-it-iv project is to develop *in vitro* test strategies for predicting the sensitising potencies of compounds, which will be able to fully replace the use of animals in safety testing (www.sens-it-iv.eu).

Due to the complex mechanisms involved in the development of sensitisation to chemicals, it is likely that no single *in vitro* test will suffice as an alternative to animal methods. Sens-it-iv attempts to overcome these limitations by exploring innovative approaches and integrating existing knowledge on the cellular and molecular mechanisms involved in chemical allergy.

The project aims to develop a detailed understanding of the roles played individually and collectively by ECs and DCs in the induction of contact sensitisation and respiratory sensitisation. The strategy will be to use a variety of *in vitro* methods to identify the important cellular and molecular events that are causally associated with the induction of allergic sensitisation. For this purpose, use will be made of freshly-isolated ECs (both respiratory epithelium and epidermal cells) and of DCs and DC lines. In each instance, all the cells used will be characterised fully with respect to phenotype and function, to allow for detailed descriptions of appropriate *in vitro* approaches. ECs and DCs, either alone or in combination, will be used to explore induced changes in gene expression, protein production and/or functional characteristics that might serve as potential markers for sensitising chemicals. For respiratory sensitisation, there will be an additional focus on the *ex vivo* functional description of these cells, by using lung slices, including the identification of the cell types, cell–cell interactions and markers involved in the acquisition of sensitisation. Sens-it-iv will also characterise, in detail, the interactions between DCs and T-lymphocytes that result in the activation, proliferation and differentiation of allergen-specific responses. Of particular importance will be descriptions of the functional sub-populations of T-lymphocytes that play decisive roles in sensitisation. In this context, a major goal will be to determine to what extent the characteristics of T-lymphocyte responses can be used as a basis for identifying and characterising sensitising hazards.

## Conclusions

Chemical respiratory allergy remains an important occupational health issue, and a real challenge to toxicologists. Although a considerable investment has been made in exploring opportunities to develop methods for hazard identification and characterisation, there are, as yet, no approaches that have received general acclaim, and no validated methods are available. Nevertheless, as discussed in this report, progress is being made, and, with continued commitment and a sustained investment in the relevant areas of research, it should prove possible to develop improved tools for safety assessment. Among the areas and approaches where progress might be expected to be made, are: an increased understanding of the characteristics of immune and inflammatory responses induced by chemical respiratory allergens; the further development and refinement of (Q)SAR models; the development of *in vitro* methods, including cell-based assays, for hazard identification; and the exploitation of advances made in the safety assessment of skin sensitisation that might be used in concert with, or as the basis for, novel approaches for the assessment of respiratory sensitisation potential.

The other major observation to derive from these deliberations has been an appreciation that, in this area, there is a particular need for close and continuing interactions among the various stakeholder disciplines. There is no doubt that progress will be greatly facilitated by informed dialogue and the sharing of expertise and experience among physicians and other occupational health professionals with interests in occupational asthma, epidemiologists, chemists, biologists, pathologists, immunologists with expertise in chemical allergy, and toxicologists who are charged with providing the effective hazard identification and risk assessment of chemical respiratory allergens.

## Recommendations

1. A structure–activity relationship model, based on a recent report by Jarvis *et al*. (147), should

be further explored as a method for the hazard identification of chemical respiratory allergens.

2. The utility of the LLNA (or possibly the reduced LLNA) as part of a tiered approach to respiratory sensitisation safety assessment of chemicals, should be further explored.

3. Opportunities should be sought for exploiting, for the benefit of respiratory allergy safety assessment, the progress that is being made (within the Sens-it-iv project and elsewhere) in the development of alternative methods for skin sensitisation testing. Among the potentially relevant approaches are peptide binding assays and cell-based methods.

4. There is a preliminary report that some contact and respiratory chemical allergens display different protein binding properties with respect to soluble and cell-associated proteins (49). These observations should be confirmed and extended.

5. There are some preliminary indications that contact and respiratory chemical allergens provoke some selective changes in gene expression in lymphoid cells *in vivo*. Investigations aimed at characterising selective gene expression signatures by contact and respiratory allergens should be expanded. There may also be opportunities to consider the application of –omics technologies to a variety of model systems.

6. Irrespective of which candidate approaches for safety assessment emerge, it would be valuable to determine the sequential, algorithmic use of different test methods, in order to maximise their utility and efficiency.

7. A second ECVAM-sponsored workshop, on *Progress in the Development of New Approaches to the Identification of Chemical Respiratory Allergens*, should be organised, to review progress against these recommendations.

8. Wherever possible, closer interactions and sustained working relationships should be fostered between the relevant stakeholder disciplines, including: clinicians and occupational health professionals with interests in occupational asthma; epidemiologists; chemists; biologists; pathologists; immunologists with expertise in chemical allergy; and toxicologists who are charged with providing more-effective hazard and risk assessment of chemical respiratory allergy.

## Acknowledgement

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