



Assessing the potency of respiratory allergens: Uncertainties and challenges

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ABSTRACT

In contrast to skin sensitisation, there are no accepted tests for the identification of chemicals or proteins with the potential to cause sensitisation of the respiratory tract. Although progress has been made, the assessment of respiratory sensitisation potential remains associated with significant challenges and uncertainties. Nevertheless, there is interest in determining whether it is possible to assess the relative potency of respiratory sensitisers. The second Adaptation to Technical Progress (ATP) to the EU Classification, Labelling and Packaging (CLP) Regulation recently introduced changes to criteria for classification and labelling of chemicals and preparations, bringing it in line with the 3rd revision to the UN Globally Harmonised System of Classification and Labelling of Chemicals (GHS). Among other things, the second ATP introduces sub-categories for respiratory and skin sensitisers, discriminating between strong sensitisers and other sensitisers. Here we examine whether such categorisation of protein and/or chemical respiratory allergens is realistic and/or feasible. For this purpose comparisons have been drawn with skin sensitisation, where potency categorisation has now been widely accepted and successfully integrated into the regulatory process. The conclusion drawn is that, on the basis of the currently available information, potency categorisation for respiratory sensitisers is premature and could potentially be misleading.

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1. Introduction

It is well established that certain chemicals and proteins have the potential to cause allergic sensitisation of the respiratory tract that may translate into respiratory allergy and asthma given sufficient, and normally repeated, exposure. A wide variety of proteins and certain classes of chemicals are associated with sensitisation of the respiratory tract and respiratory allergy.

Therefore, the predictive identification of substances that are able to cause respiratory sensitisation is one of the primary endpoints in regulatory guidelines and proposed regulations, including, for example: Registration, Evaluation, Authorisation and Restriction of Chemicals, Regulation Number 1907/2006 in EU (REACH), Biocidal Product Directive 98/8/EC in EU (BPD), Occupational Safety & Health Administration in USA, Health Canada, The Canadian Environmental Protection Act (CEPA), General Rules for Preparation of Precautionary Labels for Chemicals (GB 15258-2009) in China, the Australian Safety and Compensation Council, The National Industrial Chemicals Notification and Assessment Scheme in Australia (NICNAS). Internationally, the trend is towards adoption of the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) that prescribes for all toxicity endpoints the test methods and strategies available for their interpretation (United Nations, 2009).

For respiratory sensitisation the GHS text is very similar to that of the Dangerous Substances Directive (DSD, Directive 67/548/EEC) and REACH that followed (Commission of the European Communities, 2006). In 2009, the United Nations (UN) issued a third revised edition of GHS and in 2011 the European Commission issued the second Adaptation to Technical Progress (ATP) implementing the 3rd UN GHS revision (Commission Regulation, 2011). In the context of the objectives of this article it is relevant to consider the related GHS text from the 3rd revision of GHS and second ATP and this is provided below.

2. Classification criteria for substances

2.1. Respiratory sensitisers

2.1.1. Hazard categories

Respiratory sensitisers shall be classified in Category 1 where sub-categorisation is not required by a competent authority or data are not sufficient for sub-categorisation.

Where data are sufficient and where required by a competent authority, a refined evaluation according to paragraph below allows the allocation of respiratory sensitisers into sub-category 1A, strong sensitisers, or sub-category 1B for other respiratory sensitisers.

Effects seen in either humans or animals will normally justify classification in a weight of evidence approach for respiratory

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Table 1
Hazard category and sub-categories for respiratory sensitisers.

Respiratory sensitiser	
Category 1	A substance is classified as a respiratory sensitiser (a) If there is evidence in humans that the substance can lead to specific respiratory hypersensitivity and/or (b) If there are positive results from an appropriate animal test
Sub-category 1A	Substances showing a high frequency of occurrence in humans; or a probability of occurrence of a high sensitisation rate in humans based on animal or other tests. Severity of reaction may also be considered
Sub-category 1B	Substances showing a low to moderate frequency of occurrence in humans; or a probability of occurrence of a low to moderate sensitisation rate in humans based on animal or other tests. Severity of reaction may also be considered

sensitisers. Substances may be allocated to one of the two sub-categories 1A or 1B using a weight of evidence approach in accordance with the criteria given in Table 1 and on the basis of reliable and good quality evidence from human cases or epidemiological studies and/or observations from appropriate studies in experimental animals.

2.2. Human evidence

Evidence that a substance can lead to specific respiratory hypersensitivity will normally be based on human experience. In this context, hypersensitivity is normally seen as asthma, but other hypersensitivity reactions such as rhinitis/conjunctivitis and alveolitis are also considered. The condition will have the clinical character of an allergic reaction. However, immunological mechanisms do not have to be demonstrated.

When considering the human evidence, it is necessary for a decision on classification to take into account, in addition to the evidence from the cases:

- (a) The size of the population exposed;
- (b) The extent of exposure.

The evidence referred to above could be:

- (a) clinical history and data from appropriate lung function tests related to exposure to the substance, confirmed by other supportive evidence which may include:
 - (i) In vivo immunological test (e.g. skin prick test);
 - (ii) In vitro immunological test (e.g. serological analysis);
 - (iii) Studies that may indicate other specific hypersensitivity reactions where immunological mechanisms of action have not been proven, e.g. repeated low-level irritation, pharmacologically mediated effects;
 - (iv) A chemical structure related to substances known to cause respiratory hypersensitivity.

- (b) Data from one or more positive bronchial challenge tests with the substance conducted according to accepted guidelines for the determination of a specific hypersensitivity reaction.

Clinical history should include both medical and occupational history to determine a relationship between exposure to a specific substance and development of respiratory hypersensitivity. Relevant information includes aggravating factors both in the home and workplace, the onset and progress of the disease, family

history and medical history of the patient in question. The medical history shall also include a note of other allergic or airway disorders from childhood, and smoking history.

The results of positive bronchial challenge tests are considered to provide sufficient evidence for classification on their own. It is however recognised that in practice many of the examinations listed above will already have been carried out.

3. Animal studies

Data from appropriate animal studies which may be indicative of the potential of a substance to cause sensitisation by inhalation in humans may include:

- (a) Measurements of immunoglobulin E (IgE) and other specific immunological parameters, for example in mice;
- (b) Specific pulmonary responses in guinea pigs.

It is worth mentioning that a footnote to existing and older regulatory texts on respiratory sensitisation notes that no validated in vivo models are available, whilst recognising that data from such studies might provide useful information.

4. Potency and allergic sensitisation: some general considerations

Materials that have an intrinsic capacity to induce sensitisation (of the skin, or of the respiratory tract) are defined as representing sensitisation “hazards”. This indicates that the material possesses this particular intrinsic toxicological property. No human health effect will occur unless there is sufficient, and normally repeated, exposure of potentially susceptible individuals. However, it has long been recognised that not only does the human population vary significantly with respect to their susceptibility to sensitising materials, and the likelihood that sensitisation will be acquired, but also that those materials themselves display considerable heterogeneity. Most commonly, that heterogeneity is manifest in terms of the relative potency of a sensitiser, and indeed in the case of contact allergens it is believed that relative skin sensitising potency varies by up to five orders of magnitude (Gerberick et al., 2005; Kern et al., 2010). Relative potency, therefore, represents an important, and indeed vital, component of the risk assessment/risk management process (Basketter, 2008). Thus, for a potent sensitiser, only relatively low levels of exposure will be required for the acquisition of sensitisation, whereas with a very weak sensitiser, much higher levels of exposure will be necessary. Naturally, in this context “high” and “low” are only relative terms that serve to describe the overall extent of exposure with regard to volume, duration and maybe also frequency.

Against this background it is relevant to consider briefly skin sensitisation and what lessons have been learned from that form of allergy regarding potency assessment.

5. Skin sensitisation potency – what have we learned?

Even in some of the earliest work on human and guinea pig predictive tests for skin sensitisation, efforts were made to characterise the potency of chemicals identified as skin sensitisers (Kligman, 1966; Magnusson and Kligman, 1970). However, these tests were not ideal for potency assessment due to the fact that they were specifically designed for hazard identification, and the potency categories used (*weak, moderate, strong*, etc) were largely arbitrary and could not be validated against any existing well defined human categorisation based on either experimental evidence or clinical observation (Basketter et al., 2005a). Furthermore, such categories

did not inform either regulatory toxicology, or safety assessment more broadly.

The advent of the mouse local lymph node assay (LLNA) changed this situation (Kimber et al., 2001). This method provides an objective and quantitative readout of skin sensitising activity that is based on measurement of proliferation induced in draining lymph nodes following topical exposure of mice to a test chemical. By the end of the 20th century, the LLNA had been validated in terms of hazard identification. A 3-fold or greater increase in proliferation compared with concurrent vehicle treated controls had been shown to be equivalent in predictive capacity to the guinea pig methods which had preceded it (Gerberick et al., 2000; Dean et al., 2001). However, the existence within the assay protocol of a sensitisation induction dose response meant that it was also possible to interpolate the results to deliver a measure of the concentration of a sensitizer required to give exactly a 3-fold stimulation. This was termed the EC3 (estimated concentration causing a 3-fold increase) value (Kimber and Basketter, 1997; Basketter et al., 1999). The robustness, reproducibility and general utility of this assay and of this readout over time, and between laboratories, has been well documented (reviewed in Basketter et al., 2007). Critically, however, it is the extent to which the LLNA made it possible to compare this robust metric with measures of relative skin sensitising potency based on human experience that has proven of greatest value (Basketter et al., 2000; Ryan et al., 2000; Gerberick et al., 2001).

Initial considerations of differences between weaker and stronger skin sensitizers was developed through an examination of the EC3 values for hexylcinnamal (weak) and 2,4-dinitrochlorobenzene (DNCB; strong) (Kimber and Basketter, 1997). For this pair of skin sensitizers, EC3 values differed by more than two orders of magnitude. When a larger dataset was examined it was found that EC3 values covered almost five orders of magnitude, from 0.002% to 90% (Gerberick et al., 2005; Kern et al., 2010). Initial efforts to compare LLNA EC3 values with human data made use of extensive clinical expertise and experience, and showed that, as expected, low EC3 values correlated well with sensitizers known to be potent in man, whereas high EC3 values were usually only associated with weakly sensitising effects in humans (Basketter et al., 2000; Gerberick et al., 2001; Ryan et al., 2000). Subsequently, LLNA EC3 values were compared with threshold levels from human predictive testing (Griem et al., 2003; Schneider and Akkan, 2004; Basketter et al., 2005b). Most recently, this approach has been extended to a comparison of mouse and human thresholds for just over 100 known skin sensitizers (Basketter and McFadden, *in press*). Collectively, the results indicate clearly that there is a strong correlation between EC3 values derived from the LLNA and skin sensitisation potency in humans that is sufficient to permit derivation of a useful predictive system (Safford et al., 2011). Certainly EC3 values offer the opportunity for the identification and categorisation of those skin sensitising chemicals that are particularly potent and that therefore represent a significant human health risk (Basketter et al., 2005b; Kimber et al., 2003; van Loveren et al., 2008; Loveless et al., 2010).

Given the above, perhaps the most important use of skin sensitisation relative potency data, largely in the form of the LLNA EC3 value, is for quantitative risk assessment (Api et al., 2008). Detailed discussion of this topic is outside the scope of the present article. However, the opportunity has been acknowledged by regulatory toxicology (United Nations, 2009). As described above, there has been provision made under GHS for sub-categorisation of both skin sensitizers and respiratory sensitizers. However, a key difference is that in the case of skin sensitisation information is provided on how to interpret results from guinea pig tests and from the LLNA to facilitate this potency categorisation. For the LLNA, an EC3 value of >2% leads to sub-category 1B, indicating weaker sensitisation; all others are classified as 1A. Although the detail is not provided here,

even guinea pig test data are, in some circumstances, also capable of providing a basis for sub-categorisation, although the approach is somewhat more complex and more uncertain.

Before considering how lessons learned from skin sensitisation serve as a basis for exploring opportunities for potency classification of respiratory allergens it is relevant first to consider some broader themes.

6. Some general toxicological perspectives

In any toxicological context, effective risk assessment demands the accurate identification of hazards and an appreciation of potency; this usually requires an understanding of what level of exposure to a particular material is required to elicit adverse health effects. As indicated above, the aim of this article is to consider whether it is feasible currently to characterise and categorise chemical and protein respiratory allergens on the basis of their relative sensitising potency. In reality, accurate estimations of potency are best founded on the availability of tests or biomarkers that are known to correlate causally and quantitatively with the end point/toxic effect of interest. As described above, potency assessments for skin sensitisation using the LLNA are based upon an understanding that lymphocyte turnover in draining lymph nodes correlates quantitatively with the acquisition of sensitisation. As this metric forms the endpoint of the LLNA the opportunity exists, therefore, to deploy this assay for potency assessment as well as hazard identification. The important point is that assessment of relative potency often flows from the availability of well characterised methods for hazard identification. In the context of this article it is important, therefore, to acknowledge that there are currently no widely endorsed predictive test methods for the identification of either protein or chemical respiratory allergens.

The failure to develop an accepted test method for respiratory sensitisation is in large part attributable to the fact that there remains uncertainty regarding the important biological and biochemical processes through which sensitisation is achieved. This is true particularly with respect to chemical respiratory sensitizers. For instance, although it is well established that sensitisation of the respiratory tract by protein allergens is dependent upon IgE antibody responses, there is no general consensus about the requirement for IgE antibody for respiratory sensitisation by chemicals (Kimber et al., 1998; Kimber and Dearman, 2002; Isola et al., 2008).

Given the different challenges posed by chemical and protein respiratory allergens we will consider these two classes of sensitiser under separate headings.

7. Potency assessment of chemical respiratory allergens

There remains a general uncertainty and some confusion about the exact nature of the immunological events and processes that result in allergic sensitisation of the respiratory tract to chemicals. By analogy with protein respiratory allergy the expectation might be that IgE antibody will play a pivotal role. The difficulty is that it has not been possible to establish a good correlation between the presence of hapten-specific IgE antibody in plasma and signs or symptoms of chemical respiratory allergy (Kimber et al., 1998, 2007; Kimber and Dearman, 2002, 2005; Isola et al., 2008). Thus, although for all known chemical respiratory allergens it is possible usually to identify some patients that have detectable IgE antibody, the overall correlations are not strong. With some chemical respiratory allergens, and in particular with the diisocyanates, it is commonly the case that only a fraction of symptomatic patients have demonstrable plasma IgE antibody (Cartier et al., 1989; Cullinan, 1998; Tarlo, 1999; Tee et al., 1998; Vandenplas et al., 1993).

It may be, however, that the association between symptoms of chemical respiratory allergy and IgE antibody is much closer than the evidence cited above would indicate. For instance, there are very substantial technical hurdles to measuring accurately IgE antibody specific for a hapten. Moreover, it is possible that there may be more than one species of hapten-protein conjugate driving sensitisation (Campo et al., 2007). Assay systems rely on the availability of appropriate hapten-protein conjugates as substrates, and both the choice of protein, and the degree of substitution of that protein by hapten, have important influences on specificity and sensitivity. The result is that IgE antibody may be missed. In addition, it has been pointed out that very reactive chemicals (such as the diisocyanates) may react rapidly and extensively with OH, SH and NH₂ groups on proteins and that this will add further to the difficulties of identifying specific IgE antibody (Kimber and Dearman, 2002). Overlaying those technical difficulties is the fact that the levels of IgE antibody in plasma will decline steadily following the last exposure to chemical, and therefore the timing of sample collection will also impact on the ease with which IgE antibody is identified.

The above considerations might lead to the conclusion that there is, in reality, a much stronger correlation between chemical respiratory allergy and IgE antibody than is acknowledged currently. The argument would then be that IgE antibody might represent a legitimate biomarker of sensitisation that could be used for both the identification of chemical respiratory allergens, and determination of relative potency. This line of reasoning would suggest that an animal model in which the ability of chemicals to provoke hapten-specific IgE antibody would be an appropriate basis for safety assessment. However, as described above, there would be significant technical challenges to be negotiated to ensure provision of hapten-protein conjugates suitable for use as substrates in the IgE antibody detection systems. Furthermore, for each chemical tested there would be required one or more unique hapten-conjugates. Even if those technical challenges could be solved, and it were possible to identify chemical respiratory allergens as a function of their ability to elicit in experimental animals IgE antibody responses, then that would not necessarily imply that the level of IgE antibody produced would serve as a measure of relative potency. For that purpose it would probably be necessary to attempt to define the level of exposure required for the induction of a predetermined level of plasma IgE antibody.

While all of the above might be theoretically legitimate, in practice there is no general acceptance that IgE antibody is a universal marker of sensitisation of the respiratory tract by chemicals, and there remain important technical problems to solve in using hapten-specific IgE antibody as a readout for either hazard identification or relative potency.

In acknowledgement of problems associated with identifying hapten-specific IgE antibody for chemical allergens, an attempt was made to develop a mouse assay using induced changes in the total serum concentration of IgE (rather than the presence of specific IgE antibody) as a readout for respiratory sensitisation potential. The assay was called the 'Mouse IgE Test' and was based upon observations in BALB/c strain mice that topical exposure to chemical respiratory allergens, but not to contact allergens, resulted in an increase in total levels of serum IgE (Dearman et al., 1992; Hilton et al., 1995, 1996). The belief was that, as well as inducing specific IgE antibodies, chemical respiratory allergens also caused non-specific adjuvant-like effects resulting in an elevation of total levels of IgE immunoglobulin (Dearman et al., 1992). As a potential approach to hazard identification, this was attractive because the same endpoint (total serum levels of IgE) could be used for all chemicals, and there was no need for the preparation of hapten-protein conjugates. In the event, it was found subsequently that the mouse IgE test was not without problems and,

in particular, did not travel well, insofar as some inter-laboratory variations were recorded (Dearman et al., 1998). Nevertheless, even if induced changes in total serum IgE had proved to be a robust and reliable endpoint for the identification of chemical respiratory, there is no evidence to demonstrate that this metric would show an association with relative sensitising potency.

While a universal mandatory role for IgE antibody in the pathogenesis of chemical respiratory allergy and occupational asthma remains controversial, there is a more general consensus that respiratory sensitisation to chemicals is associated with a selective T helper 2 (Th2)-type immune response (Herrick et al., 2003). This led to the speculation that it might prove possible in mice to identify chemical respiratory allergens, and to distinguish these from contact allergens, on the basis of the quality of immune response induced. More specifically, the hypothesis was that chemical allergens of different types (contact and respiratory) would provoke discrete patterns of cytokines (and antibody production) reflective of the development of selective Th cell responses. This proved to be the case and resulted in the design of a novel approach to hazard identification that became known as cytokine profiling or cytokine fingerprinting (Dearman and Kimber, 1991, 1992, 1999, 2001; Dearman et al., 1997).

It is not necessary here to describe the procedural details of the assay used for cytokine profiling. The important point is that following repeated exposure of BALB/c strain mice to a test chemical the phenotype of induced cytokine production by draining lymph node cells is characterised. The cytokines measured are Th1-type (interferon γ and interleukin [IL]-12) and Th2-type (IL-4, IL-5, IL-10 and IL-13). Specifically, the approach seeks to determine whether a test chemical drives the development of either a selective Th1-type, or a selective Th2-type, immune response on the basis of the balance between type 1 and type 2 cytokine production. Thus, for instance, in this approach chemical respiratory allergens are defined as those where the elaboration of Th2 cytokines is greater than that of Th1 cytokines.

In our hands cytokine profiling has proven to be an effective method for the identification of chemical respiratory allergens, and for distinguishing these from contact allergens. It is important to emphasise, however, that the readout is comparative in nature, rather than absolute, and for that reason it is not clear how this approach could be translated into a method for assessing relative potency. It would not be feasible, for instance, to determine the amount of chemical required for the production of a predetermined level of expression of a specific cytokine because the method relies on interpretation of patterns of cytokine production, rather than the absolute amount of any one molecule.

The conclusion drawn, therefore, is that mouse tests that have been proposed for hazard identification of chemical respiratory allergens, and that are predicated on characterisation of aspects of induced immune responses, do not lend themselves currently, at least in their standard configuration, to assessment of relative sensitising potency.

It is relevant, therefore, to explore whether other proposed strategies for hazard identification may be of any greater utility. The other broad category of approach, that in fact pre-dated the initiatives outlined above, is based upon the attempted sensitisation of test animals (guinea pigs or rats) followed some time later by an inhalation challenge (or some other form of challenge to the respiratory tract). In such assays the potential of a chemical to induce respiratory sensitisation is assessed as a function of challenge-induced pulmonary reactions. Such methods may incorporate also consideration of other factors, such as for instance, antibody production, but the main readout is the elicitation by challenge of respiratory hypersensitivity reactions (Botham et al., 1989; Karol, 1983; Karol et al., 1985; Pauluhn, 2003; Sarlo and Clark, 1992).

It is now well established that sensitisation of guinea pigs and rats to chemical respiratory allergens, such that reactions can be elicited following subsequent pulmonary challenge, can be effected by intradermal or topical exposure (Botham et al., 1989; Pauluhn, 2003; Rattray et al., 1994). Simple topical exposure to the test material is certainly easier and more straightforward than is attempting sensitisation by inhalation that was the norm in earlier models. Topical exposure would certainly facilitate the conduct of dose response analyses at the induction stage of sensitisation in such models. The difficulty, however, is the readout of measuring challenge-induced pulmonary reactions. There remains a debate about the most effective way of measuring pulmonary hypersensitivity reactions in guinea pigs and rats. But even if a particular metric were found to be the most suitable and most reliable, then the direct comparison of chemicals with respect to their relative sensitising potency would be a very substantial undertaking using induction/respiratory challenge models. There would need to be a systematic approach to comparisons between chemicals, keeping constant a large number of important variables (including the age, strain and sex of animals, and the methods used for atmosphere generation and dosimetry). Specialised inhalation facilities would be required, and the need for test substance and the costs would both be very considerable. The view currently is that although dose response analyses can be, and are, performed in these induction/challenge models, they are not suitable for the routine characterisation of the relative respiratory sensitising potency of chemical respiratory allergens.

Thus far the relevance for potency assessment of proposed animal models has been explored. For completeness, it is appropriate also to question whether there are presently available any alternative approaches to hazard identification, based either on *in vitro* systems or on considerations of structure activity relationships (SAR), that might inform the relative potency of chemical respiratory allergens. There are as yet no *in vitro* methods for hazard identification that are of proven value (Roggen et al., 2008), and certainly there are no models presently that address questions of potency. Similarly, there has been interest in the development of SAR paradigms for the identification of respiratory allergens, but none of these has found wide application, or has yet been formally validated (Enoch et al., 2010; Seed and Agius, 2010).

Before leaving considerations of what experimental approaches might be available to provide information on the relative potency of chemical respiratory allergens, there is one final avenue to explore.

There is now available evidence that most, if not all, chemicals that are able to cause sensitisation of the respiratory tract test are positive in the LLNA (Dearman et al., *in press*). Thus, although among human populations chemical sensitisers show some selectivity with regard to the type of allergy that will be acquired by susceptible subjects, both contact and respiratory allergens will elicit clear positive results in the LLNA. This is, of course, a reflection of the fact that both classes of allergens are able to cause the activation of T lymphocytes and to provoke proliferative responses in draining lymph nodes. This raises the question of whether, in common with contact allergens, it might be possible to assess the relative potency of respiratory sensitisers as a function of activity in the LLNA, and specifically from derived EC3 values. In theory at least this appears legitimate, insofar as the vigour of immune responses to chemical respiratory allergens, and the extent to which sensitisation will be acquired, is likely to be influenced by the degree to which T lymphocytes are activated and proliferate. However, although this approach has some merit, it has to be acknowledged that in fact little is known of correlative relationships between T cell activation and sensitisation of the respiratory tract. Nevertheless, of all the opportunities explored above, and in the absence of anything better, it could be argued that potency classification of chemical respiratory allergens on

the basis of EC3 values derived from LLNA responses is the approach best equipped to guide judgements about relative potency. It has to be said, however, that the arguments in support of this strategy are largely conjectural and are unlikely currently to gain traction within the scientific community.

In the absence of experimental systems that are able to provide information regarding the relative potency of chemical respiratory allergens it is necessary to view what guidance may be available from clinical experience.

In principle clinical experience of occupational asthma should be able to inform an appreciation of relative potency. When EC3 values from the LLNA were being compared with clinical judgements of the relative skin sensitising potency of panels of contact allergens it certainly proved possible to derive some appreciation from practicing dermatologists of rank order according to potency based upon experience in the clinic (Basketter et al., 2000; Gerberick et al., 2001). However, this is by no means an exact science. When considering allergic disease in humans it is extremely difficult to disentangle sensitising potency from considerations of prevalence and severity, both of which factors are determined at least as much by conditions of exposure (the extent, frequency and duration of exposure) as they are by the intrinsic potency of the sensitising chemical. Thus, weak allergens can be common allergens if there is sufficient opportunity for exposure of individuals. In this respect the contact allergen nickel provides an illustrative example. In predictive testing, nickel is a relatively modest allergen in terms of its potency, but in many geographies, e.g. Western Europe and the USA, it is the most common cause of allergic contact dermatitis. The high incidence of nickel allergy is a reflection of ubiquitous contact with the metal rather than its intrinsic potency. Similarly, even weak allergens can cause severe hypersensitivity reactions if the conditions of exposure have been such to permit the acquisition of a high level of induced sensitisation. The most recent evidence (from the largest ever meta-analysis of elicitation studies) indicates that although induction potency varies very widely, the relative potency for elicitation varies little from the weakest to the strongest contact allergens, as judged by the minimum elicitation thresholds for a range of seven substances (Fischer et al., 2011).

It is the case therefore that judgements of relative sensitising potency predicated on either the incidence of allergy to that particular chemical, or the severity of reactions seen, can be extremely misleading. This is not to suggest that it is not possible to inform assessments of potency from clinical experience, but the important point is that informed clinical judgements in this respect demand some knowledge of the exposure conditions from which sensitisation resulted.

The conclusion is that there are presently no experimental systems or predictive tests suited to the characterisation of the relative potency of chemical respiratory allergens. Nor is clinical experience likely to be informative because in many circumstances an appreciation of exposure conditions will be lacking. For this reason the position currently is that it is not possible to derive useful assessments of relative potency that would support an informed categorisation of chemical respiratory sensitisers.

8. Potency assessment of protein respiratory allergens

There is a wide range of proteins that are able to cause sensitisation of the respiratory tract and occupational asthma. These include proteins of bacterial, fungal, plant and animal origin that are encountered in various work place settings (Sarlo and Baccam, 2007). Although the focus of our attention here is on respiratory allergy it should be noted that proteins that have the ability to cause allergic sensitisation may be associated also with allergy in other

tissues and organ systems, one example being gastrointestinal symptoms associated with allergy to food proteins.

In contrast to the position with chemical allergy, there is more certainty about the mechanisms through which protein allergens induce sensitisation. It is clear that in the case of protein IgE antibody usually plays a pivotal role. However, it would be inappropriate to assume that there is little to learn about protein allergy. There is much that we do not yet understand and, for example, it is still not clear why some foreign proteins are immunogenic but not allergenic (that is able to elicit IgG, but not IgE antibody responses), while others are both immunogenic and allergenic.

There have been two main strategies for the identification and characterisation of protein allergens. The first is predicated on the ability of protein allergens to elicit IgE (or equivalent) antibody responses, the second is to assess the ability of proteins to induce and provoke respiratory hypersensitivity reactions. Often the two approaches have been employed in tandem using guinea pigs or mice (Hilton et al., 1994; Karol et al., 1989; Kawabata et al., 1986; Ritz et al., 1993; Robinson et al., 1996, 1998; Sarlo et al., 1991; Sarlo and Karol, 1994). There have been important developments and achievements, but nevertheless there is, as yet, no formally validated method, and there is no single approach that is without drawbacks.

In the case of protein respiratory allergy the measurement of induced IgE antibody responses is an attractive endpoint for hazard identification. The ability of a protein to provoke a vigorous IgE antibody response in an appropriate animal model should be regarded as a clear sign of the potential for allergenicity. Moreover, and in contrast to the situation with chemical sensitisers, there is of course no requirement for hapten-protein conjugates as substrates for assessment of antibody responses to protein allergens.

There are, however, a number of potential problems with the use of IgE responses as a readout for respiratory sensitising hazards. One issue is that it has traditionally proven difficult to detect or measure IgE antibody in guinea pigs, frequently a species of choice for studies of respiratory sensitisation. A broader issue is the way in which specific IgE antibody is measured and evaluated. There is so far no consensus regarding the specific design of such animal studies, or how the induction route, time, dose and frequency of immunisation will impact on the outcome. A classical approach, which in some ways remains the gold standard, is passive cutaneous anaphylaxis (PCA). In this method serum is prepared from the test (immunised) animal and injected intradermally into a recipient of the same species. The recipient is then injected intravenously with a solution of the relevant antigen together with Evan's Blue Dye. If the serum from the test animal contained IgE antibody then this will associate, via specific plasma membrane receptors, with mast cells in the skin. The introduction of the antigen will cause membrane bound IgE antibodies to cross-link which in turn will lead to mast cell activation and degranulation with the release of a variety of inflammatory mediators, including vasoactive amines. The increased vascular permeability caused by the latter mediators will allow dye extravasation with a resultant bluing of the skin. The blue patches seen in the skin of challenged recipients are indicative of the presence of specific IgE antibody in the test serum.

It will be readily apparent that the PCA assay is demanding technically. It is subject to significant variability and therefore requires relatively large numbers of animals. Nor does PCA lend itself readily to consideration of dose response or measurement of the strength (titre) of IgE antibody responses. Indeed, practical experience has indicated that PCA in the guinea pigs requires that triplicates are required (Dr. N. Berg, personal communication). Not unexpectedly, therefore, there has been enthusiasm for replacing the PCA with ELISA (enzyme-linked immunosorbent assay) methods for measuring specific IgE antibody. However, ELISA assays for IgE are not easy to engineer and vary significantly in quality

and reliability. The main difficulty is that the provocation of IgE antibody responses will almost always be accompanied by IgG antibody production. The challenge from a technical perspective is that normally IgG antibodies will be produced in substantially larger amounts than IgE antibody, possibly by a factor of between 100 and 1000. In an ELISA assay IgG and IgE antibodies in the test serum sample will effectively be competing for antigenic sites on the protein substrate. There are, of course, potential solutions to avoid or minimise these issues but they are frequently technically challenging and can impact the sensitivity of the ELISA. Taken together, experience suggests that it is possible to measure specific IgE antibodies, but that the methods used are frequently technically demanding.

Accepting that the elaboration of IgE antibodies is a clear indication of a sensitising hazard, and leaving aside questions of the technical challenges inherent in the accurate measurement of such antibodies, there is one further issue that warrants consideration here. It is now well documented that the level of IgE antibody found within the plasma of sensitised subjects does not necessarily correlate closely with the clinical manifestations of allergy, or with the severity of symptoms. Food allergy provides an example where subjects with clearly demonstrable serum IgE antibody of the relevant specificity may not mount clinical reactions to the food proteins to which they are sensitised, and where in many instances the severity of clinical reactions does not correspond with levels of IgE antibody (Buck et al., 2010). This raises some questions about the general utility of specific IgE antibody *per se* as a biomarker of overall clinical reactivity.

Despite the apparent lack of a clear quantitative relationship between serum IgE antibody levels and likely clinical responsiveness to the relevant allergen, for the purposes of safety assessment using experimental animals IgE antibody is probably the most important metric. Thus, IgE antibody is the most reliable biomarker of sensitisation, and probably also represents the only practical readout for considerations of the relative potency of sensitising proteins. Assuming that specific IgE antibody induced in an experimental animal model can be measured accurately and reliably, then the vigour of IgE responses and the titre of serum IgE antibody levels, probably will give the clearest indication of potency, although it has to be borne in mind that a single point measurement of titre prohibits any consideration of the potentially important role of the kinetics of the induced immune response. For a systematic investigation of the relative potency of sensitising proteins there would be a need to define, for a range of materials, the level of exposure in a standardised experimental system required for the elicitation of a predetermined titre of IgE antibody. That is the theory; the practice would be considerably more challenging and would require a substantial investment. There would be a need to define a standard experimental system and to confirm that truly accurate measurements could be made of IgE antibodies to a wide range of proteins. This could be done, but it has not yet been accomplished in a systematic way. It is worth noting how sharply this contrasts with skin contact sensitisation to chemicals, where, notwithstanding ethical concerns, a substantial body of human experimental (i.e. not diagnostic) data on potency has been produced and used to calibrate potency predictions from the LLNA (e.g. Basketter et al., 2005b).

It is unlikely that the measurement of challenge-induced respiratory reactions would add anything of value (over and above IgE antibody production) for the assessment of sensitising potency. Nor, are there currently available any *in vitro* methods that could inform relative potency. Although there is substantial clinical experience of respiratory sensitisation to proteins, the same problems exist that were described above for chemical respiratory allergens in translating that clinical experience into knowledge of relative potency.

9. Conclusions

The recent second Adaptation to Technical Progress (ATP) to the EU Classification, Labelling and Packaging (CLP) Regulation introduces sub-categories for respiratory and skin sensitizers, discriminating between strong sensitizers and other sensitizers. Such categorisation seems feasible for skin sensitizers, where potency categorisation has been accepted and successfully integrated into the regulatory process. In contrast, for protein and chemical respiratory allergens no models exist currently even to identify sensitisation hazards, rendering sub-categorisation by this means impossible.

At a research level, for determination of the relative potency of respiratory sensitising proteins a practical approach (and probably currently the only feasible approach) is assessment of the titre of IgE antibody elicited in a standardised animal model. However, while in theory this is presently the most attractive strategy, translating this into a technically viable solution is not an easy undertaking and there are a number of important technical issues that would need to be addressed and resolved. Perhaps chief among these is the absence of human benchmarks, since there is no satisfactory way of determining the intrinsic human respiratory sensitising potency using clinical evidence.

Against this background it is premature to suggest that there is a clear way forward to measure reliably the relative respiratory sensitising potential of protein and chemical allergens.

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