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Behaviour of chemical respiratory allergens in novel predictive methods for skin sensitisation

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Abstract

Asthma resulting from sensitisation of the respiratory tract to chemicals is an important occupational health issue, presenting many toxicological challenges. Most importantly there are no recognised predictive methods for respiratory allergens. Nevertheless, it has been found that all known chemical respiratory allergens elicit positive responses in assays for skin sensitising chemicals. Thus, chemicals failing to induce a positive response in skin sensitisation assays such as the local lymph node assay (LLNA) lack not only skin sensitising activity, but also the potential to cause respiratory sensitisation. However, it is unclear whether it will be possible to regard chemicals that are negative in *in vitro* skin sensitisation tests also as lacking respiratory sensitising activity. To address this, the behaviour of chemical respiratory allergens in the LLNA and in recently validated non-animal tests for skin sensitisation have been examined. Most chemical respiratory allergens are positive in one or more newly validated non-animal test methods, although the situation varies between individual assays. The use of an integrated testing strategy could provide a basis for recognition of most respiratory sensitising chemicals. However, a more complete picture of the performance characteristics of such tests is required before specific recommendations can be made.

1. Introduction and background

Chemical respiratory allergy, associated with asthma and rhinitis, is an important occupational health issue (Kimber and Wilks, 1995; Bakerly et al., 2008; Kenyon et al., 2012; Feary et al., 2016). Probably no more than 80 low molecular weight chemicals have been confirmed as having the potential to cause allergic sensitisation of the respiratory tract, among some of the better characterised being diisocyanates, acid anhydrides, chloroplatinate salts, and certain reactive dyes (Baur, 2013; Baur and Bakehe, 2014).

From a toxicological perspective, chemical respiratory allergy remains problematic, and the challenges have been highlighted in a number of review articles (Holsapple et al., 2006; Kimber et al., 2007; 2014a;b; Boverhof et al., 2008; Isola et al., 2008; Cochrane et al., 2015; North et al., 2016). One of the key issues is that, despite a keen interest in this area for more than 3 decades, there are still no validated, or even widely accepted, methods available for the identification and characterisation of chemical respiratory allergens. That does not imply a complete lack of progress, however. In fact, a variety of strategies (based on the use of mice, rats and guinea pigs models, *in vitro* approaches, and on the identification of structural alerts) have been considered (Botham et al., 1988; Griffiths-Johnson and Karol., 1991; Satoh et al., 1995; Hilton et al., 1996; Dearman and Kimber, 2001; Arts et al., 2008; Pauluhn, 2008; Lalko et al., 2011; Enoch et al., 2012), but none of these has gained traction within the scientific community as a preferred method, and none has been viewed as being of sufficient merit to warrant progression to a formal validation process.

To a large extent the failure to identify an agreed method for the identification of chemical respiratory allergens is due to uncertainty about the mechanisms through which sensitisation of the respiratory tract to chemicals is acquired. In particular, there is a continuing debate about whether, and to what extent, IgE antibody is implicated. This issue has been discussed at some length in the literature (Kimber and Dearman, 2002; Kimber et al., 2014a; b), and will be addressed again later.

Against this background it has been necessary to adopt alternative measures to help ascertain the presence or absence of respiratory sensitisation hazards. One approach has been to explore whether the behaviour of chemicals in predictive animal tests for skin sensitisers is able to inform consideration of respiratory sensitisation potential. In this context, it is the mouse local lymph node assay (LLNA) (Kimber and Basketter, 1992; Basketter et al., 2002; Kimber et al., 1994; 2002) that has been the main focus of attention. However, results derived from guinea pig predictive tests – the guinea pig maximisation test (GPMT) (Magnusson and Kligman, 1969) and the occluded patch test (Buehler, 1965) - are relevant also.

The LLNA is a fully validated method for the identification of skin sensitising chemicals. The assay is based on an understanding that the acquisition of skin sensitisation requires, and will be associated with, the activation and proliferation of allergen-responsive T lymphocytes. Such T cell responses take place in regional lymph nodes draining the sites of exposure to a chemical allergen, and it is upon these events that the LLNA is based (Kimber et al., 2011). Subsequently, it has become apparent that, in addition to skin sensitisers, chemicals that are known to cause sensitisation of the respiratory tract elicit positive responses in the LLNA (Dearman et al., 2013). Moreover, it has been found, where data are

available, that (with a single possible exception [piperazine]) chemical respiratory allergens also test positive in the GPMT (Dearman et al., 2013).

At face value it might be considered that these data are unexpected. That is, chemical respiratory allergens are known to favour sensitisation of the respiratory tract, and are most commonly associated clinically with occupational respiratory disease. Nevertheless, there is good reason to suppose that, at least in animal models of skin sensitisation, chemical respiratory allergens will elicit positive responses. To explore why this is the case it is necessary to review briefly some basic features of immune responses to chemical allergens.

Although with many chemical respiratory allergens there are reports of cases of allergic contact dermatitis (ACD), there is a clear preference for sensitization of the respiratory tract (Kimber et al., 2011), and in some instances, such as with phthalic anhydride, chemical respiratory allergens appear to have no potential to cause skin sensitisation (Basketter and Kimber, 2016).

It is now believed that the basis for the selectivity of chemical allergens to elicit preferentially either skin sensitisation associated with ACD, or sensitisation of the respiratory tract associated with occupational asthma, is governed by the quality of immune response elicited. That is, contact allergens have been shown preferentially to induce the development of selective Th1-type T cell responses, whereas chemical respiratory allergens favour the induction of preferential Th2-type responses (reviewed Kimber et al., 2011). It must be appreciated that this is something of an over-simplification, insofar as other factors and other functional subpopulations of T lymphocytes also play important roles. However, the fact remains that such differential selectivity exists and is a pivotal factor in the elicitation by contact and respiratory allergens of different qualities of immune response,

and different forms of allergic disease. Although much of the evidence for the induction by chemical allergens of distinct T cell responses derive from studies in rodents (Dearman et al., 1995; 1996; 2005, Van Och et al., 2002; Kimber et al., 2011), there is evidence that the same pattern and relationships pertain in humans (Ouyang et al., 2013; Newell et al., 2013; Kimber et al., 2014c).

Chemical respiratory allergens therefore favour the development of selective Th2-type immune responses. As such responses, in turn, promote IgE antibody production, it appears reasonable to speculate that IgE responses play a pivotal role in sensitisation of the respiratory tract to chemical allergens, and the elicitation of respiratory allergic reactions. Certainly, IgE antibody is known to play a pivotal role in allergic sensitisation of the respiratory tract to proteins. However, the role played by IgE antibody in chemical respiratory allergy remains uncertain. The main cause of that uncertainty is that in a significant number of cases of chemical respiratory allergy (and in particular respiratory allergy associated with diisocyanates) it has not been possible routinely to identify allergen-specific IgE antibody in the plasma of symptomatic subjects (Cullinan, 1998; Kimber et al., 1998; 2010; Liu and Wisnewski, 2003).

It has been argued elsewhere (Kimber and Dearman, 2002; Kimber et al., 2011; 2014a), that there is good reason to suppose that there is a much closer relationship between IgE antibody and chemical respiratory allergy than is currently appreciated. Nevertheless, uncertainty resulting from the difficulty of detecting specific antibody in some patients with occupational asthma has prevented the acceptance of methods based on measuring IgE antibody responses. Despite a continuing debate about the relevance of IgE antibody, there is developing more of a consensus that Th2 cell responses play an important, and probably

mandatory, role in sensitisation of the respiratory tract to chemical allergens (reviewed in Kimber et al., 2014b).

The important point that emerges from this situation is that, irrespective of whether the important effector mechanism is Th2 cells or IgE antibody (the production of which is dependent upon Th2 cells), effective sensitisation of the respiratory tract will require the activation, proliferation and differentiation of allergen-responsive T lymphocytes. This being the case, it therefore should come as no surprise that chemical respiratory allergens will test positive in the LLNA where a positive response is characterised by the activation and proliferation of lymphocytes, including T cells, in draining lymph nodes. That is, that the ability to stimulate T cell responses is a common feature of contact and respiratory allergens. Where these classes of allergens differ is not in their ability to induce T cell activation, but the differentiation pathway that will be adopted in driving the development of selective Th1-type, or selective Th2-type, immune responses. It is known that chemical respiratory allergens also test positive in the GPMT due to the elicitation of a T cell response (Dearman et al., 2013).

In conclusion, therefore, there is a sound mechanistic basis for the elicitation by chemical respiratory allergens of positive responses in the LLNA (and other animal models of skin sensitisation). The clear implication is that the LLNA is not able to discriminate between contact and respiratory allergens. However, the benefit of this lack of discrimination is that a chemical that fails to test positive in the LLNA can be safely regarded as lacking not only skin sensitising properties, but also the ability to cause allergic sensitisation of the respiratory tract and occupational asthma. This paradigm has proven very useful in

providing reassurance that chemicals that test negative in the LLNA can be eliminated from consideration as potential respiratory allergens.

However, times are changing, and there are currently three validated alternative test methods for skin sensitisation hazard identification that do not require the use of animals (OECD, 2015a;b; 2016). In addition there are several other *in vitro* skin sensitisation tests that are in various stages of development, and some of these are nearing potential validation (Reisinger et al., 2015; Ezendam et al., 2016).

These issues were discussed at a recent Workshop convened by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC). This workshop titled 'Chemical Respiratory Allergy: Clinical Information and How to use it and Improve it' took place in Madrid on October 27th and 28th 2016. It was agreed that although the innovations resulting in the availability of novel non-animal test methods are to be welcomed and applauded, it is necessary to consider whether this new wave of *in vitro* test methods can be used in the same way as the LLNA for elimination from consideration as potential respiratory allergens of chemical that tests negative. The analyses below flowed from discussions during that Workshop.

The behaviour of chemical respiratory allergens in skin sensitisation test methods is an important consideration, and suggests that it should be an integral part of the validation process to establish the behaviour of chemical respiratory allergens in candidate methods for skin sensitisation. The argument is not that predictive tests for skin sensitisation should, or should not, be able to detect chemical respiratory allergens. (Indeed if a test were shown not to identify respiratory allergens then that might represent a valuable basis for distinguishing between different forms of chemical allergens). The important point is that it

is essential to have an understanding of how respiratory allergens behave in novel methods for skin sensitisation in order that decisions can be reached about whether the potential for respiratory sensitisation can be discounted in the event of a negative test.

The performance in this regard of three recently validated test methods for skin sensitisation is considered below.

2. The new wave: non-animal alternative tests for skin sensitisation

There are a number of novel non-animal approaches for skin sensitisation in development, and some in relatively advanced stages of development. However, our attention here will focus exclusively on the three methods that have recently been validated, and for which there are now available OECD guidelines (OECD2015a; b; 2016).

This focus does not imply that those methods that are currently in development do not have merit. Rather the decision to consider only those methods that have been formally validated is pragmatic, insofar as it is for these tests that richer databases are available.

It is also relevant to consider one other general point. There is a current view that it is unlikely that any single non-animal test method for skin sensitisation will have the performance characteristics required for accurate hazard identification. As a consequence, there have been several initiatives to explore how data derived from two or more individual methods can be used in concert as the basis for an integrated testing strategy (Tsujita-Inoue et al., 2014; Van der Veen et al., 2014; Jaworska et al., 2015; Takenouchi et al., 2015; Urbisch et al., 2015; Macmillan et al., 2016; Strickland et al., 2016; Ezendam et al., 2016). If one or

other integrated testing platform is shown to provide a sound basis for hazard identification then it will of course be important to consider how chemical respiratory allergens perform in that system. Although it is currently premature to explore in a systematic way the ability of integrated platforms to identify respiratory sensitising chemicals, further reference to this is made below.

The individual methods that will be considered here are: the Direct Peptide Reactivity Assay (DPRA) (Gerberick et al., 2004; 2007; OECD, 2015a), the KeratinoSens and LuSens (ARE-Nrf2 Luciferase) test methods (Emter et al., 2010; Ramirez et al., 2014; Natsch and Emter, 2016; OECD, 2015b), and the human Cell Line Activation Test (h-CLAT) (Ashikaga et al., 2006; Sakaguchi et al., 2006; Nukada et al., 2012; OECD, 2016).

The DPRA seeks to evaluate the ability of test chemicals to form stable associations with host protein; hapten-protein conjugate formation being a mandatory step in the acquisition of skin sensitisation. In practice, the assay is based upon measuring the depletion of heptapeptides incorporating either a cysteine or lysine following incubation with the test chemical (Gerberick et al., 2004). The DPRA is perhaps the only recently developed skin sensitisation test method where there have been systematic evaluations of the activity of chemical respiratory allergens (Lalko et al., 2012; Dik et al., 2016).

The second approach considered here comprises the KeratinoSens and LuSens assays, which in common with the DPRA, are based upon measurement of the electrophilic activity of chemicals. In this case such evaluations are made using a system employing a keratinocyte cell line. These cells contain a luciferase reporter gene under the control of an antioxidant response element (ARE) which senses and responds to protein-reactive chemicals (Emter et al., 2010; Natsch and Emter, 2016; Ramirez et al., 2014).

Finally, h-CLAT employs a different strategy evaluating instead the ability of test chemicals to induce the elevated expression of activation membrane markers on a human monocytic leukaemia cell like that is used as a surrogate for dendritic cells (DC) (Ashikaga et al., 2006; Sakaguchi et al., 2006). It is well established that cutaneous DC play important roles in orchestrating the acquisition of skin sensitisation to chemicals (Kimber et al., 2011).

For each of the in vitro methods mentioned above, the literature has been searched to identify all the known chemical respiratory allergens that have been evaluated. Similarly, the literature has been searched in the same way to provide the in vivo evidence in the form of results in the LLNA. All the literature sources are identified in the data compilation which appears as Table 1.

3. The activity of chemical respiratory allergens in validated alternative skin sensitisation test methods

The data available for consideration of the performance of chemical respiratory allergens in novel skin sensitisation test methods are summarised in Table 1.

This compilation serves to confirm the conclusions drawn previously that all known chemical respiratory allergens tested in the LLNA elicit a positive response. It is this relationship that provides the basis for concluding that chemicals which fail to test positive for skin sensitisation in the LLNA can also be discounted with regards to possession of respiratory sensitising activity (Dearman et al., 2013).

It will be apparent from Table 1 that there is already available a relatively rich database for the activity of chemical respiratory allergens in the DPRA. Of those allergens that tested positive in the LLNA only two tested in the DPRA (piperazine [diethylenediamine] and ethylenediamine) failed to induce positive responses, perhaps due to their requirement for metabolic activation which is lacking in this peptide assay. Although these data suggest that the DPRA may provide a relatively reliable (but incomplete) basis for the identification of respiratory sensitising chemicals, there are gaps in the available information. In particular, there is as yet no published information available on the activity of chloroplatinate salts in the DPRA (Table 1).

The data available for tests performed with KeratinoSens/LuSens or with h-CLAT are less rich, and in many instances there is no information about the performance of certain classes of chemical respiratory allergens in these assays. Thus, there is no information available for the activity of platinum salts or reactive dyes in either method, nor are there any data on the activity of diisocyanates in h-CLAT. These would be serious omissions if there were a need to determine whether these test methods have the ability to identify chemical respiratory allergens. There are, however, some data available for the activity of acid anhydrides, a class of chemical commonly associated with occupational asthma. Even here the data are incomplete, but it is instructive that KeratinoSens/LuSens do not recognise hexahydrophthalic anhydride, maleic anhydride, phthalic anhydride or trimellitic anhydride. The performance of h-CLAT with acid anhydrides is better, but nevertheless phthalic anhydride tests negative. Of course, hydrolysis of anhydrides in the aqueous systems of these in vitro assays may be part of the explanation for such unexpectedly negative results.

It is instructive to consider the possible reasons for some respiratory allergens being missed by these tests. One possible reason is that the key event on which the method is based does not provide a sensitive readout for certain classes of chemical. This is, of course, a possibility, but it is not readily apparent why, for instance, the acid anhydrides that test negative in the KeratinoSens/LuSens approach elicit positive responses in the DPRA. Although the former test is *in vitro* and the latter *in chemico*, both methods are based upon measurement of the inherent electrophilic activity of chemicals. The other possibility is, of course, that certain lipophilic chemicals are particularly difficult to deliver effectively into aqueous culture systems. This is an abiding challenge for many cell culture based toxicity assays. However, although it is always appropriate when using culture based methods to consider whether negative results might be attributable to poor delivery of the test chemical, this alone cannot explain the results summarised in Table 1 for the acid anhydrides. All but one of these chemicals (phthalic anhydride) was positive in h-CLAT; a method that also relies on delivery of chemical into an aqueous culture medium.

Taken together it would appear that – with the exception of ethylenediamine and piperazine - the DPRA mirrors the activity of chemical respiratory allergens in the LLNA. The major unknown element here currently is the performance of chloroplatinate salts in the DPRA, and it would be of some interest to see how they behave. There are also some other allergens where data are available from the LLNA, but not the DPRA. Nevertheless, it might be that the DPRA, with certain limitations, has the potential to identify most respiratory sensitising chemicals.

There can be much less certainty about the likely value of KeratinoSens/LuSens and h-CLAT in this respect. There are some important gaps in the available data and the failure of the

former approach to identify most acid anhydrides would be a major concern. The performance of h-CLAT with the acid anhydrides is better, but there are no data at all regarding the activity in this assay with diisocyanates, reactive dyes or platinum salts.

4. Discussion and recommendations

As noted earlier, predictive toxicology tests whose primary purpose is the predictive identification of respiratory sensitisers do not, as yet, exist, at least in any generally accepted or validated form. This lack renders processes of hazard identification and characterization a substantial and continuing challenge (Kimber et al, 2014b; Maier et al, 2014; Cochrane et al, 2015). Consistent with trying to fill this gap, an important driver for the analysis reported here is the historical value of negative results from *in vivo* skin sensitisation tests, such as the LLNA, in providing compelling evidence that a chemical lacks the potential to cause sensitisation of the respiratory tract. It is therefore reasonable to consider whether the *in vitro/in chemico* tests that will supersede the LLNA are also capable of providing the same information.

What emerges is an incomplete picture of the utility of these newly validated methods individually to identify chemical respiratory allergens – and therefore, by default, provide confidence in negative results being indicative of the absence of respiratory sensitising activity. However, it must be borne in mind that, in practice, it is likely that such test methods will be used and interpreted in concert. In the context of integrated testing strategies it might be that most chemical respiratory allergens will elicit positive responses

in at least one test method. The data displayed in Table 1 are in most instances consistent with this.

Irrespective of exactly how new sensitisation test methods are deployed, a question that arises from this analysis is whether there are specific studies that could be conducted to fill in some of the gaps. Among those that would probably be of greatest utility is establishing the performance of h-CLAT with one or more representatives of the following classes: diisocyanates, chloroplatinate salts, and reactive dyes. In the case of DPRA, the most significant unknown is the behaviour of chloroplatinate salts. The results of these tests would significantly improve understanding of the applicability domains (and inapplicability domains) of these tests with respect to respiratory sensitising chemicals.

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517

518 Table 1 Behaviour of chemical respiratory sensitisers in *in vitro* skin sensitisation tests

Chemical respiratory sensitiser	LLNA	DPRA	KeratinoSens /LuSens	h-CLAT
<i>Acid anhydrides</i>				
cis-1,2-Hexahydrophthalic anhydride	+	+	- ¹	+
Maleic anhydride	+	+	-	+
cis-4-Methyl-1,2,3,4-tetrahydrophthalic anhydride	+	ND	ND	ND
Phthalic anhydride	+	+	-	-
Trimellitic anhydride	+	+	-	+
Tetrachlorophthalic anhydride	ND	+ ⁴	ND	ND
<i>Amines</i>				
Chloramine T	+	+	+	+
Ethylenediamine	+	-	+	+
Piperazine (diethylenediamine)	+	- ⁴	ND	ND
<i>Aldehydes</i>				
Glutaraldehyde	+	+	+	+
<i>Diisocyanates</i>				
Diphenylmethane-4-4'-diisocyanate	+	+ ⁴	ND	ND
Hexamethylene diisocyanate	+ ¹	+ ¹	+ ¹	ND
Toluene diisocyanate	+	+ ⁴	+	ND
Isophorone diisocyanate	+ ¹	+ ¹	+ ¹	ND
<i>Platinum salts</i>				

Ammonium hexachloroplatinate IV	+	ND	ND	ND
Ammonium tetrachloroplatinate II	+	ND	ND	ND
cis-Dichlorodiammine platinum II	+	ND	ND	ND
<i>Reactive dyes</i>				
Basic Blue 99	+ ⁵	+ ⁴	ND	ND
Orange 3R Reactive Dye	ND	+ ⁴	ND	ND
Reactive Black 5	+	+ ⁴	ND	ND
Reactive Red 123	+	ND	ND	ND
Reactive Yellow 39	+	+ ⁴	ND	ND
<i>Uronium salts</i>				
2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate	+	ND	ND	ND
2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate	+	ND	ND	ND
<i>Others</i>				
Colophony/abietic acid	+	+	+	-
Cyanuric chloride	+	+	ND	ND
Piperidinyl chlorotriazine	+	ND	ND	ND
Potassium dichromate (an example of Cr VI compounds)	+ ²	ND	+ ³	+ ²
Penicillin G (an example of penicillin compounds)	+	+	-	+

519

520 The table is adapted and extended from the publication of Dearman et al, 2013. In vitro data have
521 been derived from Asturiol et al, 2016 except where indicated: ¹ Urbisch et al, 2015; ² Ashikaga et al,
522 2010; ³ Ramirez et al, 2014; ⁴ Lalko et al, 2012; ⁵ Cosmetic Ingredient Review, 2007; ND = no data.

Highlights

- Chemical respiratory allergens test positive in in vivo skin sensitisation assays
- The performance of such allergens in in vitro skin sensitisation tests is examined
- Correlations were good, but not perfect, and there remain gaps to be filled