The Development of Novel Approaches to the Identification of Chemical and Protein Respiratory Allergens

Progress made from the Conclusions and Recommendations of ECVAM Workshop 60[a]

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Background

In October 2006, the European Centre for the Validation of Alternative Methods (ECVAM) sponsored and hosted a three-day workshop to consider opportunities and challenges in the development of alternative methods for the identification and characterisation of chemical respiratory allergens. The proceedings, conclusions and recommendations of that workshop were subsequently made available as ECVAM Workshop Report 60 (1).

In April 2008, a second meeting was convened by ECVAM to consider what progress has been made in the light of the recommendations derived from the 2006 Workshop and the published report. This meeting had as a second objective — to consider whether new opportunities for the identification and characterisation of chemical respiratory allergens had emerged since 2006. In addition, the scope of the meeting was broadened to also include an examination of new and emerging approaches that may be appropriate for the evaluation of the respiratory sensitising potential of proteins.

The Identification and Characterisation of Chemical Respiratory Allergens: Progress Since 2006

The recommendations made in the report of the previous workshop (1) included the following:

— Further exploration of a model describing structure–activity relationships (SAR) for chemical asthmagens/respiratory allergens (2);

— Continued consideration of the potential utility of the murine local lymph node assay (LLNA) as part of a tiered approach to screening for chemical respiratory allergens;

— Confirmation and further extension of previous reports indicating that contact and respiratory chemical allergens show selectivity of association with cell-associated or soluble proteins, respectively (3);

— Examination of whether respiratory chemical allergens display differences from contact allergens in their association with model peptides,

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with respect to amino acid specificity and/or kinetics of interaction (4);

— Exploiting wherever possible, the extensive experimental work currently in progress, which is examining changes in the phenotypic characteristics of cultured dendritic cells (DCs) following their interaction *in vitro* with contact allergens. Specifically, the recommendation was that further consideration should be given as to whether contact and respiratory chemical allergens provoke differential effects on gene expression and protein production by DCs (5).

As reflected in the above recommendations, it is apparent that at the time of the ECVAM workshop in 2006, with the exception of the SAR model, the main focus of attention was (and still remains) the exploitation and refinement of cellular and protein-based assays that were initially developed for the purposes of skin sensitisation testing, for the purposes of chemical respiratory allergen identification. In each of these areas, investigations are continuing, but this is — in each case — work in progress, and no single approach highlighted in the workshop recommendations has yet borne fruit with regard to chemical respiratory allergy detection.

Chemicals and hypersensitivity reactions in the respiratory tract

Two very recent articles have reviewed the fact that, in contrast to the situation for skin sensitising chemicals, there are, as yet, no generally accepted methods for the identification of chemicals that result in allergic sensitisation of the respiratory tract (6–7). These reviews provide excellent summaries of current knowledge, so only brief comments will be made here.

The term *hypersensitivity reaction in the respiratory tract* is used to describe a wide variety of responses resulting from exposure to sensitising agents, notably the early and late allergic reactions, airway inflammation and airway hyper-responsiveness. It seems likely that, depending on the sensitiser, the level of exposure and the type of response provoked, only some of these symptoms may develop. Likewise, current animal models only reflect some of these pathophysiological alterations. Thus, hypersensitivity reactions in the lung are multi-factorial, and the mechanisms involved are incompletely understood.

It has been well documented that chemical sensitisation requires hapten–protein conjugate formation (8–9). Also, it has been demonstrated in animal models that chemical exposure via the skin or respiratory tract can result in sensitisation of the respiratory tract, such that pulmonary hypersensitivity reactions can be elicited by subsequent inhalation challenge (10–14). Thus, for the purposes of hazard identification, routes of exposure other than inhalation may also be relevant. Hapten–protein conjugate formation and immunological priming (sensitisation) do not necessarily lead to clinical disease in every individual.

The production of T-helper 2 (Th2) cytokines and IgE antibodies are frequently implicated in hypersensitivity reactions in the lung. While an association has been found in mice between chemical respiratory allergens and Th2 responses (15), the association of respiratory sensitisation to low molecular weight chemicals with IgE production appears to be less strong (13–14, 16). Similarly, specific IgE antibody is not always detectable in symptomatic patients with occupational asthma resulting from exposure to chemical respiratory sensitisers. For example, specific IgE is not consistently detected in individuals with asthma associated with diisocyanates (17) However, an association between specific IgE and asthma which has been induced by trimellitic anhydride (TMA) and other acid anhydrides, has been noted (18). Explanations for the inability to detect an association with IgE antibody in patients may include one or more of the following: a) IgE antibody is present, but not detected effectively in serum for technical reasons (19); b) IgE levels decline steadily following the last exposure, making timing of blood sampling an important issue; and c) mechanisms other than those involving IgE and Th2 cytokines are required for hypersensitivity reactions in the lung to some chemicals.

Although it is recognised that sensitisation to respiratory allergens can occur through the skin, some investigators have favoured, for animal studies, instillation of, or exposure to, the chemical via the respiratory tract. A problem associated with identifying chemicals that produce allergic sensitisation of the respiratory tract is the fact that many chemicals are not readily soluble in water. Thus, the application of these chemicals to the lung is more difficult than that of water-soluble substances. Farraj *et al.* (20) used intranasal instillation of TMA in acetone/olive oil in an attempt to cause a hypersensitivity reaction in the lung, but only nasal pathological effects resulted, partly because of inadequate distribution of the TMA to the lung compartment. When studying hypersensitivity reactions in the lung, species differences also appear to be critically important — for instance, with respect to mast cell responses (21). Moreover, the clinical relevance of the most frequently studied and best understood mouse model is under debate (22–23).

Short-term Opportunities: Chemical Respiratory Allergen Identification by Using Novel Methods for Identification of Skin Sensitising Chemicals

One of the purposes of the meeting was to review recent advances in the development of novel meth-

ods for the identification of skin sensitising chemicals, and to consider the possible application of these approaches to the evaluation of chemical respiratory allergens. Since animal models are available for the detection of skin sensitising chemicals that have been well validated and widely accepted, and because there has been a substantial and relatively long-term investment in alternatives research in this area, it is reasonable to speculate that advances here may accelerate the development of analogous methods for chemical respiratory sensitisers.

As has been described previously, for a chemical to cause contact sensitisation, a number of requirements must be met. These include access to the viable epidermis, reactivity with protein (or conversion in the skin to a protein-reactive metabolite) and hence the ability to form stable conjugates with proteins, elicitation of cytokine production by skin cells, the activation of DCs, and the initiation of T lymphocyte responses (24). Many chemical respiratory allergens share some of these requirements, including protein reactivity, although such chemicals may also be defined by their ability to provoke the preferential development of Th2-type immune responses (25). It has therefore been proposed that: a) some of the current tests for chemical contact allergens may in fact be indicative of a sensitising hazard *per se* (encompassing both contact and respiratory sensitisers; 1); and b) modifications of such tests may pave the way for the development of methods that can differentiate between contact and respiratory sensitising properties.

The peptide-binding assay

One method identified in the previous report (1), the peptide binding assay, which reflects the requirement for chemical sensitisers to form stable associations with proteins (4, 26). In the current assay conformation, the use of synthetic cysteine and lysine peptides, rather than conventional proteins, has the advantage that the analyses are simpler — allowing for the development of relatively high-throughput screening assays (26). Analyses of 82 chemicals (30 non-sensitisers and 52 sensitisers, including 4 known respiratory sensitisers) revealed a prediction accuracy of 89%, with all 4 respiratory sensitisers testing positive. Preliminary investigations with a wider range of chemical respiratory sensitisers suggest that peptide binding may, in fact, be a common property of these materials (27). However, there remains a need for prospective studies and additional work, to determine whether contact and respiratory sensitisers do indeed differ with respect to amino acid specificity and/or kinetics and/or affinity of binding. It must be emphasised that the development of peptide binding assays will require consideration to be given to the requirement for some chemicals to be activated metabolically. Work is in progress to examine options for including within the test configuration an exogenous metabolising system.

DC-based assays

Another *in vitro* approach for the identification of contact sensitisers that has received considerable attention, is the activation by chemical allergens of DCs, or of surrogate DC-like cells, either alone or in co-culture with other cells, such as keratinocytes (5, 28–34). Many endpoints have been examined, including the altered or induced plasma membrane expression of cell surface markers, such as CD86 or CD54, and the secretion/expression of certain cytokines or chemokines, such as interleukin (IL)-8 or macrophage inflammatory protein (MIP)-1α. There are now several independent reports on the selective candidate gene lists obtained following the culture of DC preparations with contact sensitisers and the application of microarray technology (35–38). These may provide additional biomarkers for contact sensitisation that warrant further study. In general, a relatively small number of chemicals have been examined, and few inter-laboratory comparisons have been initiated. An exception is work conducted with the human Cell Line Activation Test (h-CLAT) by using the THP-1 cell line, where two independent laboratories have performed analyses with 9 chemicals (33), and one laboratory has conducted tests with 29 chemicals (34). One potentially fruitful initiative is an activity supported by ECVAM and COLIPA (the European Cosmetic Toiletry and Perfumery Association), in which several independent laboratories will examine the responses of two cell lines (THP-1 and U937) by using the expression of CD54 and CD86 in response to a range of sensitisers as readouts. As part of this prevalidation process, it may be appropriate to determine whether such assays can also detect chemical respiratory sensitisers. In addition, skin sensitisers such as 2,4-dinitrochlorobenzene (DNCB) have an intrinsic ability to polarise adaptive immune responses toward Type 1 cytokine expression, whereas chemical respiratory sensitisers (such as trimellitic anhydride [TMA]) instead direct immune responses toward selective Type 2 cytokine expression (5). Therefore, it may be possible to modify the cell-based assays described above, in order to distinguish between skin and respiratory sensitisers on the basis of differential cytokine/chemokine expression.

It should also be noted that, in their investigations, Toebak and co-workers (5) used DCs derived from fresh peripheral blood monocytes and measured the ratio of IL-10 to IL-12 production. The selection of these cytokines is based upon the understanding that IL-10 is a characteristic Type 2

cytokine, while IL-12 is a characteristic Type 1 cytokine. For a cell line-based assay, measurements of secretions of these two cytokines must be demonstrable for the cell line of choice. MUTZ-3 is a very promising cell line for incorporation into an *in vitro* assay, since, compared to all the cell lines studied to date, it physiologically most-closely represents its *in vivo* counterpart. The MUTZ-3 progenitor cell is readily available and can be differentiated into large quantities of immature Langerhans cells (MUTZ-LC) or interstitial DCs (39). Only MUTZ-derived DCs (i.e. neither the MUTZ-3 progenitor nor the MUTZ-derived Langerhans cell) are able to secrete very low levels of IL-10 and IL-12 (unpublished data). The performance in *in vitro* assays of DCs derived from MUTZ-3 and MUTZ cells still needs to be determined.

The murine LLNA

The murine LLNA is an *in vivo* assay for the identification of skin sensitising chemicals. This was the first test to pass through the formal regulatory validation process established in the USA by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Subsequently, the ECVAM Scientific Advisory Committee (ESAC) approved the LLNA as an alternative to guinea-pig tests for the identification of skin sensitisation hazard, recognising the substantial reduction and refinement of experimental animal usage achieved by this test method (40–41). As discussed in the previous report (1), a retrospective analysis of the recently published LLNA database (42) demonstrated that a variety of chemicals known to be associated primarily with respiratory allergy in humans are universally positive in the LLNA (and in other tests for the identification of skin sensitising chemicals). Therefore, the current view is that a chemical that tests negative in the LLNA can be regarded as lacking the potential for either skin sensitisation or sensitisation of the respiratory tract.

It is appropriate to highlight the recent publication of a modified version of the LLNA (43). This modified or "reduced" LLNA (rLLNA) can be used for screening purposes when there is a need to evaluate the sensitising activity of a large number of chemicals, such as will be the case under the auspices of the EU Registration, Evaluation and Authorisation of Chemicals (REACH) system. It is proposed that the method is suitable for identifying skin sensitising hazards. However, it is important to emphasise that the rLLNA is unsuitable for the evaluation of relative potency. Thus, where hazard characterisation is required for risk assessment purposes, the standard LLNA, rather than the rLLNA, should be used. A retrospective analysis of LLNA data for 211 chemicals revealed that the rLLNA (comprising only a single high dose group, rather than the standard three dose groups, and a concurrent vehicle control), in the majority of cases, provided accurate information regarding sensitising potential, provided that care was taken to maximise application concentrations. Clearly the use of the rLLNA can, in certain circumstances, provide important animal welfare benefits (with respect to reduction), although it must be acknowledged that the rLLNA will inevitably be associated with some increased level of uncertainty. There has been no formal evaluation of the ability of the rLLNA to identify chemical respiratory allergens, although the database described by Gerberick *et al.* (42) included a small number of respiratory sensitisers, each of which would have been identified as positive on the basis of the information from the top dose group and the concurrent vehicle control group alone.

The Current Status of *In Vitro* Approaches for Assessing the Sensitising Potency of Proteins

A growing understanding of the molecular and cellular immune processes that are engaged following exposure to foreign proteins has raised expectations that, in the near future, screening tools will become available for *in vitro* sensitisation testing. Currently, two types of *in vitro* assay are envisaged. The first addresses the innate (non-specific) responses elicited in epithelial cells (ECs) and DCs following encounter with a protein. The second group of assays seeks to identify the T-cell and B-cell epitopes expressed by the protein. Finally, there are, in addition, a number of computer-based epitope mapping tools that are designed to identify T-cell and B-cell epitopes.

In vitro test formats monitoring the interaction between a compound and ECs and/or DCs have been developed. It must be stressed, however, that no validated approach for assessing the sensitising potential or potency of proteins is yet available. There is evidence that ECs can be triggered by exposure to chemicals to express surface proteins and soluble mediators *in vitro*, that are known *in vivo* to be involved in the activation, adhesion, chemotaxis, differentiation and proliferation of innate and adaptive immune cells in both skin and the lung (44–45). This has encouraged their use in the development of tests to address early events in the immune response, especially in skin and respiratory sensitisation. Biomarkers with the potential for predicting the allergenic potency of proteins have not yet been identified. However, macrophage-colony stimulating factor (M-CSF) expression by ECs has been shown to correlate with the allergenicity of proteases in mice. Similarly, granulocyte-colony stimulating factor (G-CSF) appears to represent a promising marker for

lipase allergenicity (46). These results suggest that *in vitro* EC-based test systems can provide information on the sensitising potency of proteins. These data also suggest that the allergenicity (and probably also the immunogenicity) of a protein has two aspects: i) the effects on cellular processes which result in the modification of secreted signal proteins or membrane-bound proteins, or in the lipolysis of membrane lipids; and ii) the provision of epitopes recognised by B-cells and T-cells (see below).

It is recognised that DCs provide an important link between innate and adaptive immune function via a process of maturation, that requires CD40 ligation in addition to antigen presentation and CD80/CD86 co-stimulation (47). Although much of the early work on DC maturation was performed *in vitro*, it still is not clear how these biomarkers can be exploited for evaluating the immunogenicity and allergenicity of proteins *in vitro* (44–45). An *in vitro* approach system that combines ECs and DCs within a single test system may be the tool of choice. Such immunocompetent tools are currently being developed in a larger European Commission-sponsored Integrated Framework 6 Project (Sens-it-iv: www.sens-it-iv.eu).

The central role played by macrophages in immune responses has been elegantly reviewed by Stoy (48). Chen *et al.* (49) demonstrated that, *in vivo*, proteins can enhance the production of inflammatory mediators by macrophages, as well as the accessory function of these cells. Furthermore, Radyuk *et al.* (50) reported a test system for studying the role of airway ECs and alveolar macrophages in the clearance of anthrax spores and the prevention of infection. In spite of these promising results, macrophages have so far been neglected in the development of cell-based assays for assessing the immunogenicity of compounds.

Classical epitope mapping methods have identified a significant number of T-cell and B-cell epitopes which were collected in a variety of databases. The B-cell epitope databases have been used to evaluate the predictive power of tools originally developed to analyse physicochemical and structural features from the primary structure of the study protein. Unfortunately, prediction of antigenicity from the sequence of a protein by using any of these tools, either alone or in combination, is inaccurate. T-cell and B-cell epitopes can be addressed by comparing amino acid sequence and structural similarities. It is believed that proteins having a similar structure and, at the same time, sharing a substantial part of the amino acid sequence, also share antigenic properties. The problem with this approach is that the minimal sequence similarity required to make this work still has to be defined. Finally, a number of computer-based epitope mapping tools have been demonstrated to identify T-cell and B-cell epitopes with various degrees of accuracy. For these tools to be accepted as prediction tools for research and regulation, it is imperative that they are evaluated on high-quality training and testing datasets. Furthermore, our knowledge of IgE specific epitopes is limited, or possibly non-existent, making these tools primarily useful for general immunogenicity assessment (51).

Conclusion

The purpose of this meeting was to assess: a) progress made against the recommendations deriving from the 2006 workshop, and b) whether new opportunities had emerged since then.

With respect to the progress made, it was concluded that, with the exception of the SAR model by Jarvis *et al.* (2), the test formats currently being assessed in the field of respiratory sensitisation have been adopted and refined from the field of skin sensitisation. The main focus remains on cellular and protein-based assays.

Progress in the field of *in vitro* respiratory sensitisation by chemicals is hampered by the lack of a generally-accepted animal model, the complexity of the lung, and the yet poorly understood mechanisms leading to respiratory sensitisation and hypersensitivity. Thus, the successful incorporation of methods into an accepted test strategy for assessing the respiratory sensitisation potencies of chemicals requires continued research, anchoring to *in vivo* endpoints, extensive standardisation, and collaboration between the researchers involved.

Among current activities in the area of skin sensitisation, three test methods were discussed: a) the peptide-binding assay; b) DC-based assays; and c) the LLNA. With respect to the first of these, the available data indicate that peptide binding is a requirement for both skin and respiratory sensitisers. However, there remains a need for prospective studies and additional work, to determine whether contact and respiratory sensitisers do indeed differ with respect to amino acid specificity and/or kinetics and/or affinity of binding. A variety of DC-based assay formats are being assessed with variable success. This may be explained, in part, by the endpoints that are currently used for estimating the effects of chemical exposure (e.g. CD54, CD86, IL-8). Selective gene lists may contain more-adequate markers for skin sensitisation, but these remain to be identified and their potentials have to be demonstrated. Since skin sensitisers and respiratory sensitisers trigger different types of T-cell mediated responses, it might be possible to distinguish between both classes of sensitisers by the differential genomics or cytokine/chemokine expression. Such research is ongoing, but data supporting this hypothesis are not yet available. With respect to the LLNA, it is increasingly accepted that this test is suitable for identifying skin sensitisation hazards. However, the utility of the LLNA (or its reduced

form, the rLLNA) as part of a tiered approach to the identification of respiratory sensitising hazards, has yet to be confirmed.

Some progress has been made in the assessment of the sensitising potencies of proteins, but this progress is linked to specific protein classes. A general *in vitro* test is not yet available and, as for chemical respiratory sensitisers, additional research is required to provide better understanding of the immunobiology of protein sensitisation, as well as to develop and implement an *in vitro* assay. However, it is anticipated that the allergenicity of proteins develops via the interaction of the intact protein with ECs and immune cells, concomitant with the recognition by the immune cells of T-cells and B-cell epitopes. As yet, cell-based assays, as well as computer-based assays, to assess each of these pathways are under development.

Overall, it is fair to state that no test model is yet available for assessing the sensitising potentials or potencies of respiratory allergens. Work is ongoing to provide better understanding of the biology of hypersensitivity in the lung and to translate this knowledge into *in vitro*-based and computer-based testing tools. An investment in coordinated multidisciplinary research continues to be required for making *in vitro* testing for respiratory sensitisation possible.

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