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Dendritic cells and skin sensitisation hazard assessment

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Abstract

Allergic contact dermatitis is an important occupational and environmental health disease. There is a need, therefore, to identify skin sensitisation hazard, and to assess accurately likely risks to human health. During the past 15 years very significant advances have been made in our understanding of the cellular and molecular mechanisms that serve to initiate and regulate cutaneous immune responses, including the acquisition of skin sensitisation. This has facilitated parallel advances in the identification and characterisation of skin sensitising chemicals and the development of more robust approaches to risk assessment. It is relevant to consider whether advances in immunobiology provide opportunities also for the design of alternative approaches to the toxicological evaluation of skin sensitisation, including the development of in vitro methods. Here we review the potential use of strategies based on analysis of responses induced in Langerhans cells and dendritic cells; professional antigen processing and presenting cells that are known to play pivotal roles during the induction phase of adaptive immune responses.

Keywords: Allergy; Cytokines; Dendritic cells; Hazard identification; Langerhans cells; Skin sensitisation

1. Skin sensitisation

Allergic contact dermatitis is a common health problem and many chemicals are known to cause skin sensitisation. The immunobiological mechanisms that are required for the acquisition of skin sensitisation, and for the subsequent elicitation of allergic reactions in the skin are complex and dependent upon highly orchestrated molecular and cellular interactions. Contact allergy has been the subject of comprehensive reviews (Basketter et al., 1999a; Grabbe and Schwarz, 1998; Kimber et al., 2002a; Kimber and Dearman, 1996, 1997, 1998, 2002; Rustemeyer et al., 2001; Smith and Hotchkiss, 2001), and a detailed account is unnecessary here. For the purposes of this article only a brief summary is necessary. Skin sensitisation is induced when a susceptible subject is exposed topically to the inducing chemical allergen. Exposure must be sufficient to provoke a cutaneous immune response of the vigour necessary for the development of sensitisation. Once sensitisation has been acquired then the subject will respond to subsequent contact with the inducing allergen by mounting an accelerated and more aggressive secondary immune response that in turn will precipitate a cutaneous inflammatory reaction at the site of exposure that is recognised clinically as allergic contact dermatitis.

The central event during the induction of skin sensitisation is the activation and clonal expansion of allergen-specific T lymphocytes in lymph nodes draining the site of exposure. It is this increase in the frequency of allergen responsive lymphocytes that represents the cellular basis for heightened reactivity to the inducing allergen. The stimulation of T lymphocyte responses requires that the antigenic stimulus is delivered from the skin in an appropriate form to draining lymph nodes. This is largely the responsibility of epidermal Langerhans cells (LC) and the dendritic cells (DC) into which they mature. Here we will consider the roles of LC and DC in the initiation and regulation of skin sensitisation, and how they might be exploited in pursuit of in vitro methods for skin sensitisation testing. However, as a prelude to that it is appropriate to consider very briefly the current position with respect to hazard assessment.

Guinea pigs were the original species of choice for the identification of skin sensitising chemicals. Several model systems were developed, those used most widely

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being the guinea pig maximisation test (Magnusson and Kligman, 1970) and the occluded patch test of Buehler (1965). In such tests, sensitising activity is determined as a function of challenge-induced skin reactions in previously sensitised animals. More recently, an alternative test method, the local lymph node assay (LLNA), has been developed and validated in which allergenic potential is measured on the basis of the induction, rather than the elicitation, phase of contact sensitisation. Specifically, sensitising potential is measured as a function of lymph node cell (LNC) proliferative responses induced following topical exposure of mice to test chemicals (Basketter et al., 2002; Dearman et al., 1999; Gerberick et al., 2000; Kimber and Basketter, 1992; Kimber et al., 1994, 2002c). The LLNA offers important animal welfare benefits, with respect to both reduction and refinement compared with guinea pig test methods, but there is nevertheless interest in exploring whether opportunities exist for the design of wholly in vitro approaches to hazard identification. Some of the proposed strategies have been reviewed previously (Kimber, 2000; Kimber et al., 1999a; 2001b; Ryan et al., 2001). Here we will concentrate on how an appreciation of the roles of epidermal LC in the acquisition of skin sensitisation provides the basis for one avenue of investigation.

2. Langerhans cells and the induction of skin sensitisation

The biology of LC and their contributions to the initiation of cutaneous immune responses are subjects of considerable complexity. Comprehensive accounts of LC generally, and of their roles in skin sensitisation specifically, are available (Austyn, 2001; Cumberbatch et al., 2000; Friedmann, 1981; Kimber and Cumberbatch, 1992; Kimber et al., 1998, 1999b, 2000; Maurer and Stingl, 2001). In the skin, epidermal LC can be considered to act as sentinels of the immune system, with responsibility for surveying changes in the antigenic microenvironment. In response to antigenic challenge, a proportion of local LC is stimulated to become mobilised and to migrate to draining lymph nodes bearing novel antigen for presentation to responsive T lymphocytes (Kimber et al., 2000). In fact, it is clear that LC are induced to migrate in response to cutaneous trauma irrespective of whether or not the tissue disruption is initially associated with exposure to foreign antigen (Kimber et al., 2002b).

While resident in the epidermis LC have the properties required for the effective recognition, internalisation, processing and subsequent transport of antigen. However, during the course of their journey from the skin to draining lymph nodes LC are subject to a functional maturation during which they lose the capacity to process antigen and instead acquire the characteristics of immunostimulatory DC. The cells that reach the regional lymph nodes home to, and localise within, the T lymphocyte-rich paracortical regions where antigen presentation to T lymphocytes is effected.

The pivotal roles played by LC in the development of cutaneous immune responses and skin sensitisation require highly regulated changes in phenotype and appropriate cell–cell and cell–matrix interactions. To a large extent these processes are orchestrated by chemokines and epidermal cytokines (Kimber et al., 2000). It is now clear, for instance, that in the mouse the mobilisation and migration of LC following skin sensitisation, and their subsequent localisation within lymph nodes, are dependent upon at least three epidermal cytokines: tumour necrosis factor α (TNF-α; Cumberbatch and Kimber, 1995), interleukin (IL) 1β (Cumberbatch et al., 1997; Antonopoulos et al., 2001) and IL-18 (Cumberbatch et al., 2001). Moreover, homing of trafficking LC to the paracortical regions of lymph nodes requires ligand interactions with CCR7, a chemokine receptor that is up-regulated on LC as they migrate from the skin (Forster et al., 1999). There is increasing evidence that the migration of human LC from the skin is initiated and regulated by very similar, or indeed identical, mechanisms (Cumberbatch et al., 1999; Kimber et al., 2000).

3. Assessment of Langerhans cell/dendritic cell responses in vitro

Given the importance of epidermal LC in the initiation of skin sensitisation, it appears reasonable to consider whether there exist opportunities to develop alternative approaches to hazard identification based upon chemical-induced changes in the phenotype or functions of these cells. A rationale for such a strategy was provided first by the investigations of Enk and Katz (1992) who characterised changes in epidermal cytokine gene expression induced by topical exposure of mice to contact allergens or contact irritants. The interesting observation was that some cytokines appeared to be induced or up-regulated only by skin sensitisers. Among these was IL-1β, a cytokine that in mouse epidermis is produced exclusively by LC (Enk and Katz, 1992; Enk et al., 1993). The implication was that the rapid (15 min) increase in mRNA for IL-1β associated with skin sensitisation might be attributable to a direct affect of the contact allergen on resident LC. For this reason, allergen-induced changes in the expression by cultured DC of IL-1β was viewed by delegates at a Workshop convened in 1995 by ECVAM (European Centre for the Validation of Alternative Methods) to provide one realistic approach to skin sensitisation testing in vitro (De Silva et al., 1996). There were, however, major difficulties in translating this opportunity into practice,

not least of which was the availability of suitable cell culture systems. In the epidermis, LC represent only a minority population, and their isolation and purification is technically demanding. Moreover, it is clear that following isolation LC are subject to rapid changes in phenotype. Despite these difficulties, purified LC, or LC within mixed epidermal cell suspensions, have been used by some investigators for the purposes of examining changes associated with exposure to chemical allergens (Herouet et al., 1999; Rizova et al., 1999; Verrier et al., 1999). However, there are now available methods, using appropriate cytokine cocktails, for the expansion of DC, including DC with an LC-like phenotype, from progenitor cells in blood, cord blood or bone marrow (Bender et al., 1996; Caux et al., 1992; Inaba et al., 1992; Lenz et al., 1993; Romani et al., 1994; Sallusto and Lanzavecchia, 1994).

Human peripheral blood-derived DC with the general characteristics of LC have now been used to examine in vitro the impact of exposure to chemical allergens. The cytokine cocktail used to generate such relatively immature DC from peripheral blood monocyte precursors usually comprises a mixture of IL-4 and granulocyte/macrophage colony-stimulating factor (GM-CSF). It must be acknowledged, however, that as yet there is no standard method for the generation of human DC cultures, and there is evidence that other cytokines, including for instance IL-3 and transforming growth factor β (TGF-β), are also able to influence the differentiation of monocytes into LC-like cells (Ebner et al., 2002; Guironnet et al., 2002).

Cultured DC derived from human peripheral blood were used by Reutter et al. (1997) who reported that some chemical allergens, but not a non-sensitising skin irritant (sodium lauryl sulfate; SLS), were able to provoke increases in the expression of mRNA for IL-1β. This observation was confirmed subsequently in investigations conducted in this Laboratory. DC derived from the peripheral blood of healthy donors were used. It was found that several skin sensitising chemicals, including *p*-phenylene diamine, methylchloroisothiazolinone/methylisothiazolinone and 2,4-dinitrofluorobenzene (DNFB) were all able to stimulate the increased expression of IL-1β mRNA, but only in cells from a proportion (approximately 50%) of subjects (Pichowski et al., 2000). Subsequent studies revealed that the differences observed between donors were stable with time. That is, in repeat analyses DC cultures derived from donors with a 'responder' phenotype continued to display increased levels of mRNA for IL-1β in response to DNFB. Similarly, DC from 'non-responder' donors consistently failed to exhibit increased levels of IL-1β mRNA following treatment with the same allergen (Pichowski et al., 2001). Despite evidence for stable phenotypes with respect to IL-1β responses *in vitro*, the view is that these are not necessarily of any relevance to skin sensitisation, and almost certainly do not reflect inter-individual differences with respect to the ease with which contact sensitisation will be acquired. In common with the investigations of Reutter et al. (1997), our experience was that even with DC derived from donors with responder phenotypes non-sensitising skin irritants such as SLS and benzalkonium chloride failed to stimulate an increase in IL-1β mRNA expression (Pichowski et al., 2000, 2001).

Several conclusions can be drawn from these data. The first, and probably most important, is that the direct interaction of chemical allergens with LC-like cells in vitro can cause a change in gene expression that corresponds with what is known to happen during skin sensitisation. It is of interest also that (based admittedly on very limited experience) at least some skin irritants appear not to provoke similar changes. While these observations are intriguing, it is important to acknowledge that in the context of developing an alternative approach to hazard identification there are several significant limitations. The fact that DC derived from only a proportion of donors are of a responder phenotype with respect to IL-1β poses logistical problems. Moreover, even with DC of a responder phenotype, the increases observed in IL-1β mRNA were very modest (in the order of two- to three-fold), despite the use of what are known to be very potent contact allergens. Such modest changes suggest that this particular approach would lack the sensitivity required even for a screening test.

Other investigators, using similar, but not necessarily identical, experimental designs have also failed to observe allergen-induced increases in the expression of IL-1β, or have at least failed to find consistent changes (Aiba et al., 2000; Tuschl and Kovac, 2001). However, there are phenotypic changes, other than the altered expression of IL-1β, that are known to be associated with the differentiation of LC following skin sensitisation. Investigations using human blood monocyte-derived DC have revealed that the expression of certain of these markers are in some instances altered following treatment with contact allergens. Among those that have been found to be responsive are: class II major histocompatibility complex (MHC) gene products (such as HLA-DR), co-stimulatory molecules (such as CD86) and adhesion molecules (such as CD54) (Aiba et al., 1997, 2000; Coutant et al., 1999; Hulette et al., 2002; Tuschl and Kovac, 2001).

When one considers the extent of changes that will be provoked when LC are induced to differentiate in response to skin sensitisation, it is clear that only a small fraction of potential markers have thus far been examined. It is known that the functional maturation of LC is associated with the altered expression of cytokines and chemokines, cytokine and chemokine receptors, adhesion molecules, costimulatory molecules, matrix

metalloproteinases and many other gene products, including those involved in signalling and metabolism. One illustration of the scope and scale of changes precipitated by the induced differentiation of human DC is provided by the studies reported by Le Naour et al. (2001). Using a combination of microarray transcript profiling and proteomics these investigators identified changes in gene expression and protein production following the TNF-α-induced differentiation of human blood monocyte-derived DC. A total of 255 genes was shown to be regulated during differentiation, many of which are not normally associated with the immunological properties of DC. It is not yet known to what extent cytokine-induced differentiation of DC reflects the changes to which these cells are subject following skin sensitisation. However, experiments are in progress currently to address this question, and to provide a broader view of gene expression changes induced in LC-like cells by chemical allergens (Pennie and Kimber, 2002). The hope is of course that more holistic investigations of this type will allow the identification of genes that exhibit robust changes in expression, and that may as a consequence provide more sensitive correlates of allergenic potential.

4. Mouse bone marrow-derived DC

In parallel with continuing investigations of the application of human monocyte-derived DC, we are exploring the potential utility of DC derived from mouse bone marrow. The method used is to generate immature DC from precursor cells in mouse bone marrow using GM-CSF as a growth factor (Fields et al., 1998; Inaba et al., 1992; Lutz et al., 1999). As indicated above, LC and immature DC are known to play pivotal roles in the internalisation and processing of antigen for subsequent presentation to T lymphocytes (Mellman et al., 1998; Mellman and Steinman, 2001; Thery and Amigorena, 2001), and many investigators have used mouse bone marrow-derived DC to characterise the molecular mechanisms involved (Mahnke et al., 2000; Olasz et al., 2002; Pierre et al., 1997; Santambrogio et al., 1999). Of particular relevance in the context of this article is the fact that mouse bone marrow-derived DC conjugated with the relevant hapten are able to stimulate secondary proliferative responses by T lymphocytes prepared from skin sensitised mice, and are able to induce skin sensitisation in naïve mice (Olasz et al., 2002).

In this laboratory the approach we have taken to generate mouse bone marrow-derived DC with an immature phenotype is as follows. Bones (femurs and tibiae) are collected and washed, and cells flushed from the marrow cavity with phosphate buffered saline (PBS). Cells are cultured in complete medium supplemented

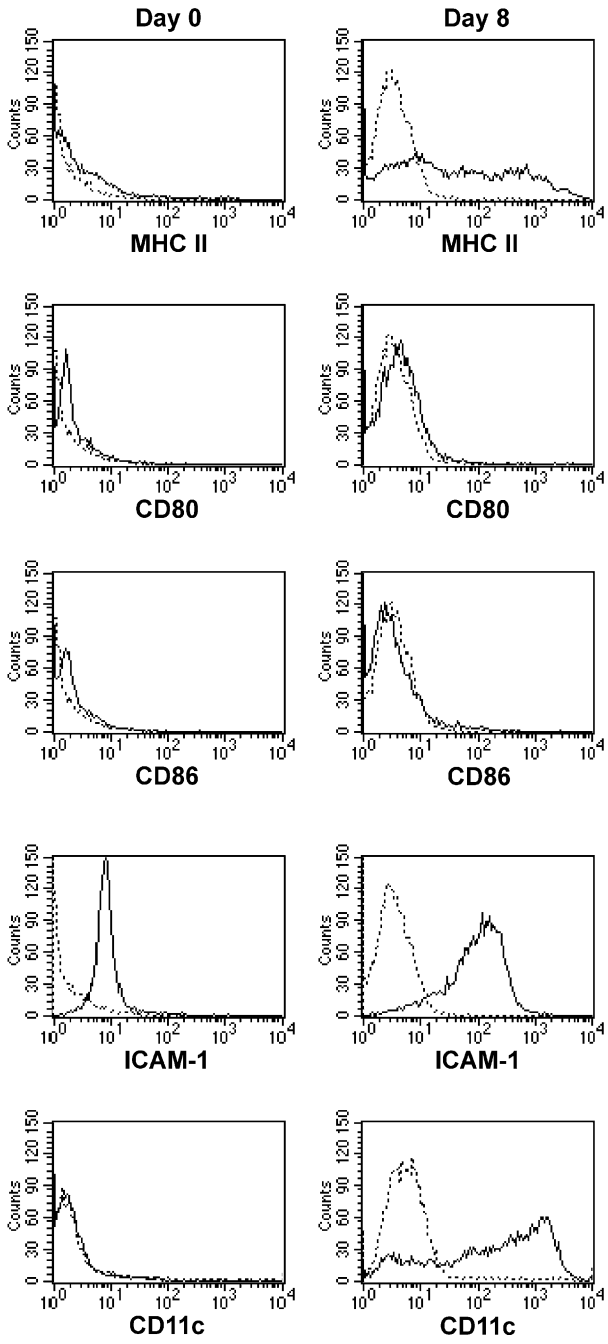

Fig. 1. Flow cytometric analysis of bone marrow-derived DC on day 0 and day 8 of culture in GM-CSF. Cells were seeded into 96-well round bottom plates (5×10^5 cells/well) and incubated with antibodies directed against I-A^d/I-E^d (MHC class II) diluted to 5 μg/ml (clone 2G9, rat IgG2a), CD80 (10 μg/ml; clone 1G10, rat IgG2a), CD86 (10 μg/ml; clone GL1, rat IgG2a) [all from BD PharMingen], ICAM-1 (CD54; 10 μg/ml, clone KAT-1, rat IgG2a, R&D Systems), or rat IgG2a control (BD PharMingen), followed by treatment with F(ab′)2 goat anti-rat IgG:FITC (1/100; Serotec). Alternatively, cells were pre-treated with rat anti-mouse CD16/32 to block Fc receptors followed by labelling with PE-conjugated hamster anti-mouse CD11c (10 μg/ml; clone HL3, BD PharMingen) or with PE-conjugated hamster IgG isotype control (10 μg/ml; clone G235-2356, BD PharMingen). Data were acquired using a FACSCalibre flow cytometer (BD Biosciences). The results displayed represent analyses of 2×10^4 cells. Bold lines = antibody staining; dotted lines = isotype control.

with GM-CSF for 8 days. Non-adherent cells (DC) are removed and characterised by flow cytometry. A representative phenotypic analysis is illustrated in Fig. 1, where DC generated following 8 days of culture in GM-CSF have been compared with the initial cell population. There is an increased expression of DC-related markers (MHC class II determinants, intercellular adhesion molecule 1 [ICAM-1; CD54], and the integrin CD11c). In contrast, and consistent with a comparatively immature DC phenotype, cells derived in this way from bone marrow progenitors do not display the membrane co-stimulatory molecules CD80 and CD86. The morphology of mouse bone marrow-derived DC is shown in Fig. 2. Using dextran linked with the fluorochrome fluorescein isothiocyanate (FITC-dextran) it has been possible to demonstrate that these cells have endocytotic activity. Preliminary experiments suggest that these immature mouse DC are able to respond in culture to skin sensitising chemicals. A representative experiment is summarised in Fig. 3 where exposure of DC to the contact allergen DNFB resulted in the increased expression of mRNA for several cytokines (IL-1β, IL-6, IL-12 and IL-18). It must be emphasised, however, that these are preliminary experiments and that experimental conditions have still to be evaluated fully and optimised. Nevertheless, experience to date is encouraging and suggests that mouse DC of an appropriate (immature) phenotype can be generated from bone marrow precursors and used to characterise changes in gene expression and protein production induced by contact allergens. Such an approach may provide a supplementary method for the identification of DC markers suitable for the identification of skin sensitising chemicals.

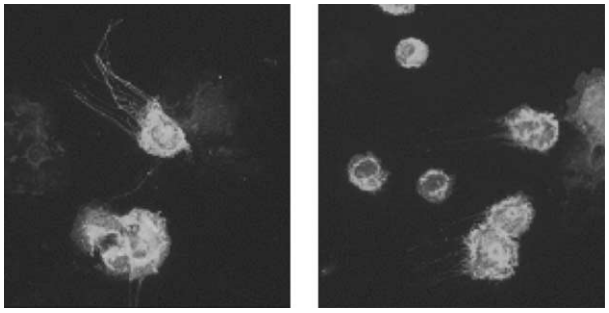

Fig. 2. Morphology of DC harvested on day 8 of culture and stained for MHC class II expression using antibodies described in the legend to Fig. 1. Original magnification 100×.

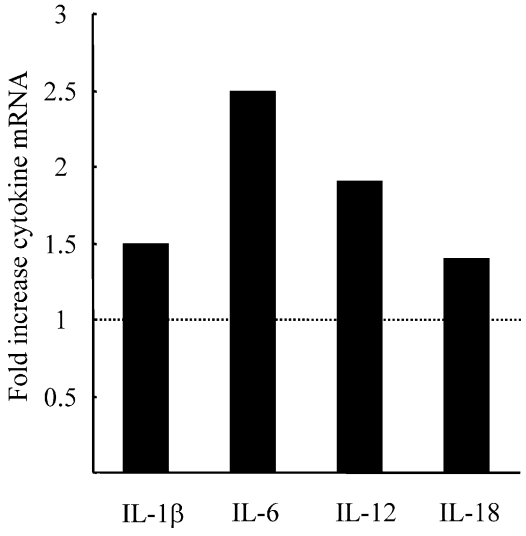

Fig. 3. Induced cytokine mRNA expression following exposure of mouse bone marrow-derived DC with a contact allergen. DC (2×10^5/ml), prepared as detailed in the legend to Fig. 1, were cultured in 24 well flat bottomed plates and treated with 2,4-dinitrofluorobenzene (DNFB; 5 nM) in 0.01% DMSO, or with the same concentration of DMSO alone, for 2 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested at room temperature and total RNA extracted TRIZOL (Invitrogen). Cytokine mRNA expression was measured by reverse transcription-polymerase chain reaction (RT-PCR) using 1 μg total RNA per reaction with amplification of between 25 and 30 cycles at 60 °C. PCR products were subjected to agarose gel electrophoresis and band intensity measured by digital camera imaging. Data from a representative experiment are shown. Cytokine gene expression was normalised against the expression of a housekeeping gene (hypoxanthine phosphoribosyl transferase) and recorded as fold increase in cytokine mRNA induced by exposure to DNFB compared with levels of mRNA associated with treatment with DMSO alone (vehicle control; dashed horizontal line).

5. Conclusions

The challenge is to harness our increased understanding of immunobiology generally, and of the mechanisms of skin sensitisation specifically, to design realistic in vitro tests that can be used in the context of toxicological evaluations. This challenge is significant, not least because the exquisite complexity of the immune system makes difficult the development of in vitro model systems that reflect accurately characteristics of adaptive immune responses. As discussed above, the initiation of a specific immune response requires cellular and molecular interactions that are tightly regulated in time and space. Nevertheless, it is clear that LC in the skin, and DC generally, play essential roles in the initial phases of immune responses, being required for the internalisation, processing and presentation of antigen. For this reason attention has focused largely, but not exclusively, on the use of such cells in attempting to recapitulate in vitro key events during the induction phase of skin sensitisation. It is possible, but not yet proven, that the judicious interpretation of DC responses in vitro may provide the basis for hazard identification in the future, or if that is not possible, a screening method for identifying potent contact allergens. However, the challenges do not end there. The accurate identification of hazard represents

only one element of toxicological evaluation. The development of risk assessments demands that an appreciation of the likely conditions of exposure is integrated with an understanding of potency, rather than simply intrinsic hazard. Measurement of relative potency using guinea pigs tests proved problematic (Kimber et al., 2001a), and the approach of choice currently is based on consideration of dose–responses induced in the LLNA (Kimber and Basketter, 1997; Basketter et al., 1999b). This strategy has been shown to provide estimates of relative potency that correlate closely with what is known of the ability of chemical allergens to induce skin sensitisation among human populations (Basketter et al., 2000; Gerberick et al., 2001). A truly valuable in vitro method for the assessment of skin sensitising activity would be required to provide some indication of relative potency. This, in turn demands that the end-point(s) chosen is known to correlate causally and quantitatively with the acquisition of skin sensitisation. The issue is best viewed from the perspective of induced increases in the expression by LC-like cells of mRNA for IL-1β. It is known that IL-1β is required for LC mobilisation and migration, and for the optimal development of skin sensitisation, but there is no evidence (as yet) that the total amount of IL-1β produced is a critical determinant of the relative sensitising potential of a chemical allergen.

While there is no doubt that some important (but fascinating) challenges need to be addressed, there is reason to believe that it will be possible in the future to develop in vitro approaches, which may not necessarily be 'stand-alone' methods, but could nonetheless contribute usefully to the evaluation of skin sensitising potential. The requirement is for a continued investment in chemical allergy research and a willingness to embrace enthusiastically the new opportunities for the design of alternative test methods that will inevitably result from an increasingly sophisticated appreciation of the mechanisms of skin sensitisation.

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