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Chemical Allergy: Translating Biology into Hazard Characterization

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The induction by chemicals of allergic sensitization and allergic disease is an important and challenging branch of toxicology. Skin sensitization resulting in allergic contact dermatitis represents the most common manifestation of immunotoxicity in humans, and many hundreds of chemicals have been implicated as skin sensitizers. There are far fewer chemicals that have been shown to cause sensitization of the respiratory tract and asthma, but the issue is no less important because hazard identification remains a significant challenge, and occupational asthma can be fatal. In all areas of chemical allergy, there have been, and remain still, intriguing challenges where progress has required a close and productive alignment between immunology, toxicology, and clinical medicine. What the authors have sought to do here is to exemplify, within the framework of chemical allergy, how an investment in fundamental research and an improved understanding of relevant biological and biochemical mechanisms can pay important dividends in driving new innovations in hazard identification, hazard characterization, and risk assessment. Here we will consider in turn three specific areas of research in chemical allergy: (1) the role of epidermal Langerhans cells in the development of skin sensitization, (2) T lymphocytes and skin sensitization, and (3) sensitization of the respiratory tract. In each area, the aim is to identify what has been achieved and how that progress has impacted on the development of new approaches to toxicological evaluation. Success has been patchy, and there is still much to be achieved, but the journey has been fascinating and there have been some very important developments. The conclusion drawn is that continued investment in research, if coupled with an appetite for translating the fruits of that research into imaginative new tools for toxicology, should continue to better equip us for tackling the important challenges that remain to be addressed.

Key Words: **skin sensitization; dendritic cells; Langerhans cells; T lymphocytes; local lymph node assay; respiratory sensitization.**

The purpose of this article is to explore toxicological aspects of chemical allergy, this being the most common manifestation of immunotoxicity in humans. Specifically, it is our aim to describe how an appreciation of relevant biological and immunological mechanisms can inform the development of new and improved approaches for hazard identification and characterization.

With those objectives in mind, it is important to recognize and acknowledge the scope and complexity of chemical allergy. In common with other forms of allergy, allergic reactions caused by chemicals develop in two stages. Following first encounter with a chemical allergen (in sufficient quantity and via an appropriate route of exposure), an inherently susceptible subject will mount an adaptive immune response that results in specific immunological priming that, in the context of allergy, is described as ''sensitization.'' If the now-sensitized subject is exposed subsequently to the same chemical allergen (again subject to amount and site of exposure), then an accelerated and more aggressive secondary immune response will be provoked that results in inflammation that is recognized clinically as an allergic reaction. The development of an allergen-induced inflammatory reaction in a sensitized subject is commonly described as an ''elicitation'' or ''challenge'' reaction.

During the course of this article, we will be considering two forms of chemical allergy. The first of these is skin sensitization resulting in allergic contact dermatitis (ACD). This is a common occupational and environmental health problem, and many hundreds of chemicals are known to have the potential to cause skin sensitization. Examples of the appearance of allergic contact dermatitic reactions are shown in Figure 1. The other form of chemical allergy that we will consider here is occupational rhinitis and asthma resulting from allergic sensitization of the respiratory tract. Fewer chemicals are known to cause respiratory allergy, but the health impact can be catastrophic.

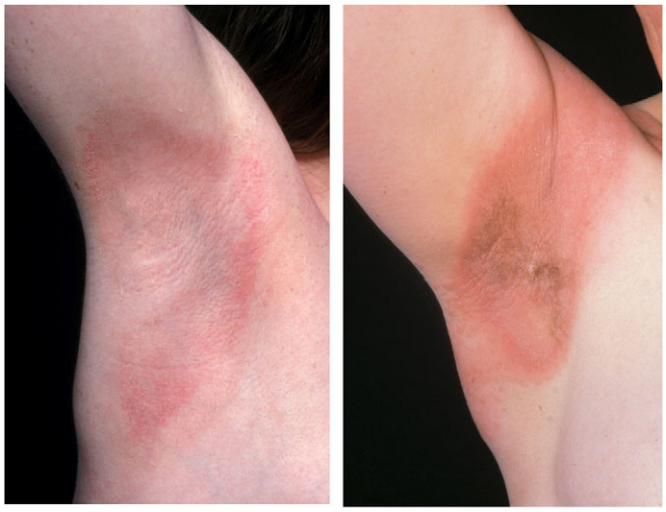

FIG. 1. ACD of axillae associated with the use of personal care products.

The acquisition of sensitization and the subsequent elicitation of an allergic reaction are complex immunobiological processes that demand multiple orchestrated cellular-molecular interactions that are highly regulated in time and space (Kimber and Dearman, 1997a). It is clearly beyond the scope of this article to catalog the immunological bases for all the processes that contribute to the development of various forms of chemical allergy, and it is necessary, therefore, to be selective in the ground that will be covered. Against that background, we have elected here to explore three specific aspects of chemical allergy, specifically, (1) the role of epidermal Langerhans cells (LC) in the development of skin sensitization, (2) T lymphocytes and skin sensitization, and (3) sensitization of the respiratory tract. These areas have been chosen because they reflect aspects of chemical allergy where an investment in more fundamental research has already paid dividends (some more significant than others) in informing hazard identification and characterization and where we believe there exist important opportunities and challenges that if addressed effectively will impact further on evolution of new testing strategies.

The authors acknowledge that within this paper, their considerations of the areas highlighted above are rather selective and far from exhaustive. For that reason and to redress a partisan view of the literature, we have, where possible, drawn attention to relevant review articles from which more thorough treatments can be sourced.

THE ROLE OF EPIDERMAL LC IN THE DEVELOPMENT OF SKIN SENSITIZATION

The skin immune system is complex. It has the role of providing protection against injury and infection, and to this end, it is essential that sentinel and surveillance functions are matched by immunoregulatory control mechanisms (Guilliams *et al*., 2010b; Loser and Beissert, 2007; Nestle *et al*., 2009; Strid *et al*., 2009). An important part of the mosaic of cutaneous immune function is the LC.

Epidermal LC (first described by Langerhans, 1868) form part of a much wider family of phenotypically diverse bone marrow-derived dendritic cells (DC) that are now recognized as being the primary cellular orchestrators of adaptive immune responses while being sited at the crossroads of the innate and adaptive immune systems (Cella *et al*., 1997; Banchereau and Steinman, 1998; Guilliams *et al*., 2010a; Toebak *et al*., 2009). Until recently, LC were considered to be the class of DC that was largely or exclusively responsible for the acquisition of skin sensitization following encounter at skin surfaces with a chemical allergen (Cumberbatch *et al*., 2000, 2003b; Kimber *et al*.,1998a, 2000).

We and others have sought to identify and characterize the signals and events that initiate and regulate the mobilization, migration, and maturation of LC during the induction phase of sensitization. Briefly and in its simplest form, the paradigm was that following encounter with chemical allergen at the skin surface, LC become activated and are mobilized. Activated LC bearing allergen then migrate from the epidermis, via the afferent lymphatics, to peripheral skin-draining lymph nodes where they localize within the paracortex. While in transit to the nodes, LC are subject to a functional differentiation such that they mature from antigen processing cells into DC-like cells that are able effectively to present antigen to responsive T lymphocytes (Kimber and Cumberbatch, 1992).

Those events, in the context of the acquisition of skin sensitization, are illustrated in Figure 2; a figure that also provides a pictorial overview of the induction and elicitation phases of ACD.

This remains an accurate description of LC function but fails to reflect the fact that LC are now considered to have a broader functional remit within the skin and that other populations of skin DC can and do contribute to the development of immune responses to chemical allergens (Kimber *et al*., 2009; Stoitzner, 2010). The morphological appearance of human LC within the epidermal matrix is shown in Figure 3, together with a diagrammatic representation of some of the main phenotypic characteristics of epidermal LC.

Irrespective of what the varied responsibilities of DC might be (and we shall return to this later), we have, in tandem with others, been able to characterize some of the important cytokine signals that drive and regulate LC mobilization and migration. In mice, there are at least three cytokines that are known to play essential roles: interleukin (IL) 1β, IL-18, and tumor necrosis factor α (TNF-α) (Antonopoulos *et al*., 2001, 2008; Cumberbatch and Kimber, 1992, 1995; Cumberbatch *et al*., 1997, 1999a, 2001, 2002). Interestingly, there is both direct and indirect evidence that the same, or at least very similar, cytokine signals are required for the mobilization of LC in human skin (Cumberbatch *et al*., 1999b, 2003a; Griffiths

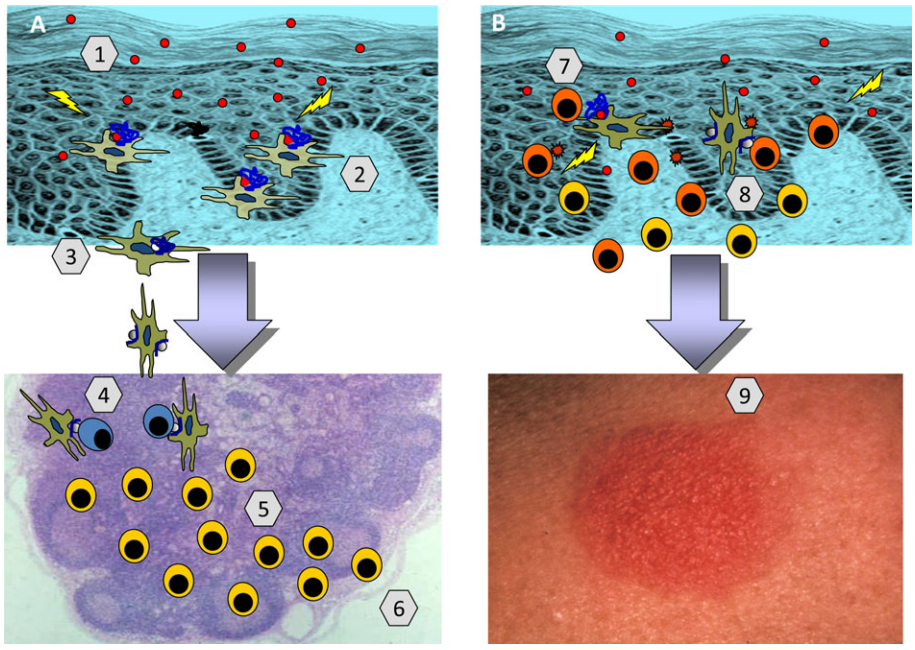

FIG. 2. The induction phase of skin sensitization (A). Chemical allergens gain access to the viable epidermis and associate in stable fashion with protein (1). There is the local release of various proinflammatory cytokines and other ''danger signals'' that are required to support immune activation and the engagement of DC (2). LC (and other cutaneous DC) are activated and recognize, internalize, and process haptenated protein. These cells transport antigen from the skin to draining lymph nodes, via the afferent lymphatics, during which time they become activated and differentiate into mature, antigen-presenting cells (3). Haptenated peptides are presented to naive, antigen-responsive T lymphocytes (4). The antigen-driven activation of responsive cells is associated with rapid turnover and selective clonal expansion of antigen-specific T lymphocytes (5). The expanded population of primed antigen-specific T lymphocytes (effector and memory T lymphocytes) disseminates into the peripheral circulation (6). At this point, sensitization has been acquired. The elicitation of ACD (B). Elicitation is triggered by exposure of the now-sensitized subject, at the same or a different skin site, to the same chemical allergen (7). Allergen-specific T lymphocytes accumulate at the site of encounter with the chemical (8). T lymphocytes become activated and are stimulated to release cytokines, chemokines, and other inflammatory mediators that act in concert to draw in other leukocytes and drive the cutaneous inflammatory reaction that is characterized by erythema, edema, and visiculation and that is recognized clinically as ACD (9).

et al., 2001, 2005). The interplay between these cytokines and the wide variety of effects they have on LC has not been established fully. However, the view presently is that there is an important early requirement for IL-18 and that this cytokine acts upstream of both IL-1β and TNF-α, maybe being necessary for sustaining increases in the levels of expression of these proinflammatory cytokines. Both IL-1β and TNF-α deliver independent, but complementary, signals directly to LC via IL-1RI and TNF-R2 receptors, respectively. These signals provoke a range of changes in LC that collectively permit them to free themselves from surrounding cells in the epidermis and equip them for the cell-matrix interactions necessary for travel to regional lymph nodes (Kimber *et al.*, 1999a, 2000; Stoitzner *et al.*, 2002; Toebak *et al.*, 2009). Important also are changes in the expression by LC of chemokine receptors, including the elevated expression of C-C chemokine receptor type 7 that is important for their homing to and localization within draining lymph nodes (Cyster, 1999; Kimber *et al.*, 2000). It is probable also that a counterregulatory influence is provided by local production of the anti-inflammatory cytokine IL-10 that is thought to inhibit, or at least downregulate, LC migration secondary to an inhibition of TNF-α production (Kimber *et al.*, 2000). A diagrammatic summary of some of the key signals and events in the mobilization, migration, and homing of LC is shown in Figure 4.

More than 20 years ago, the late Wayne Streilein came to the conclusion that for the induction of skin sensitization in mice, epidermal LC are "sufficient, but not required." His investigations revealed that under circumstances where LC were unavailable (through selective removal of the epidermis), then such sites could still support the acquisition of skin sensitization through the medium of dermal DC (Streilein, 1989). The debate about whether LC are the exclusive antigen processing/presenting cells of the skin, at least in the context of skin sensitization, was initiated again more recently with the availability of mice lacking LC and by the use of complementary experimental strategies. What has emerged is a complicated picture, but the available evidence points to an endorsement of the view expressed by Streilein that the presentation of chemical allergens within the skin immune system and the development of skin sensitization can proceed without LC (Bennett and Clausen, 2007; Bennett *et al.*, 2005, 2007; Fukunaga *et al.*, 2008; Kaplan *et al.*, 2005, 2008; Kissenpfennig and Malissen, 2006; Romani *et al.*, 2006; Wang *et al.*, 2008).

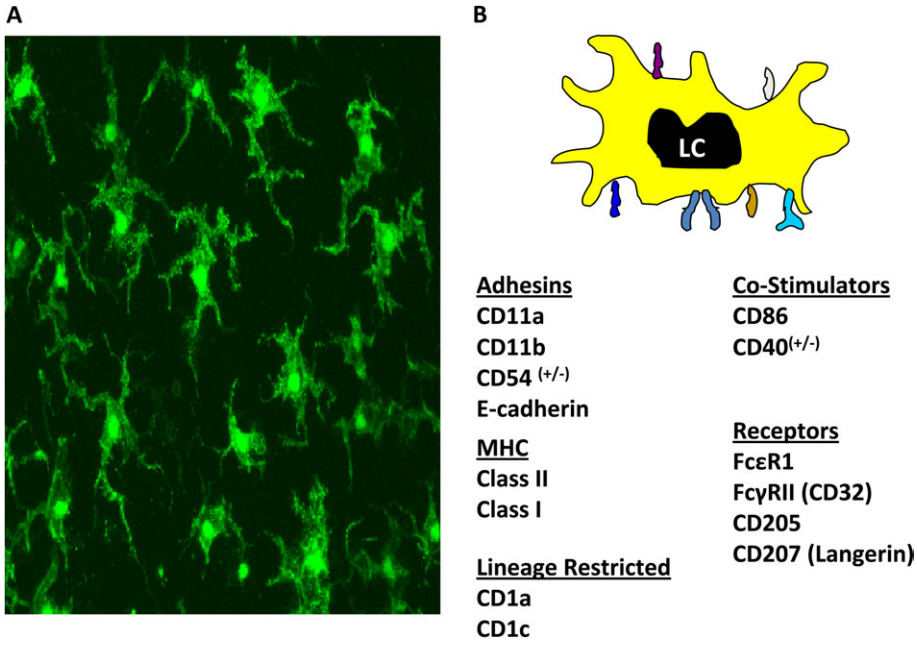

FIG. 3. Human LC within the epidermal matrix. Epidermal sheets were prepared from the normal skin of a human volunteer and stained for the expression by LC of MHC class II determinants (A). Some of the major phenotypic characteristics expressed by human LC while resident within the epidermis are illustrated diagrammatically (B).

The implication is of course that in certain circumstances, DC within the dermis can also present antigen effectively and induce sensitization. There are present within skin at least two populations of dermal DC that differ in phenotype and function, and the precise contributions these make to cutaneous immune responses are still not entirely clear (Guilliams *et al.*, 2010a; Larregina *et al.*, 1997; Nestle *et al.*, 1993; Toebak *et al.*, 2009).

Perhaps more importantly than simply confirming that within skin LC are not the sole immunostimulatory cells, studies that have used selective ablation strategies have revealed that LC may also possess immunoregulatory/immunosuppressive properties (Bobr *et al.*, 2010; Clausen and Kel, 2010; Igyarto and Kaplan, 2010; Lutz *et al.*, 2010; Stoitzner, 2010; Yoshiki *et al.*, 2009). The fact that LC are able not only to trigger cutaneous immune responses but also constrain and regulate them further emphasizes the important impact these cells will have on the development of skin sensitization. It is still not clear what circumstances and events shape the functional behavior of LC and the balance between immunostimulatory and immunoregulatory activity. However, one can speculate that among the factors that may be influential are the level of maturation of LC and their location within the skin matrix following mobilization; the nature, amount, and disposition of the inducing antigen; the local cytokine environment; and the presence of other immunomodulatory factors.

In addition to the above properties, it is clear that LC (and probably other populations of DC within the skin) are able to help shape the polarity of adaptive immune responses. This is achieved by triggering the selective development of functional subpopulations of T lymphocytes that in turn tailor the quality of immune responses to match the challenge posed by the inducing antigen (De Jong *et al.*, 2005; Klechevsky *et al.*, 2008; Liu *et al.*, 2001; Sen *et al.*, 2010; Smits *et al.*, 2005). As will be considered later, chemical allergens of different types induce in mice the development of polarized immune responses, and there is evidence that this may in part be governed via the activity of epidermal LC (Cumberbatch *et al.*, 2005).

Taken together, the evidence available indicates that epidermal LC display considerable plasticity with respect to the balance between stimulation and regulation and in their ability to promote polarized T lymphocyte responses. The picture is complicated further by the fact that there exist at least two subpopulations of DC within the dermis that also contribute to cutaneous immune responses. Notwithstanding this complexity and diversity, it is nevertheless the case that the successful acquisition of skin sensitization will be dependent upon the engagement of one or more populations of DC. Skin DC have the task of recognizing, processing, and transporting antigen in an immunogenic form for presentation to responsive T lymphocytes, and without those tasks being accomplished, skin sensitization will fail to develop.

As a consequence, there has been considerable interest in exploiting our understanding of the importance of DC in skin sensitization for the purposes of designing novel *in vitro* approaches to hazard identification. This is based on an

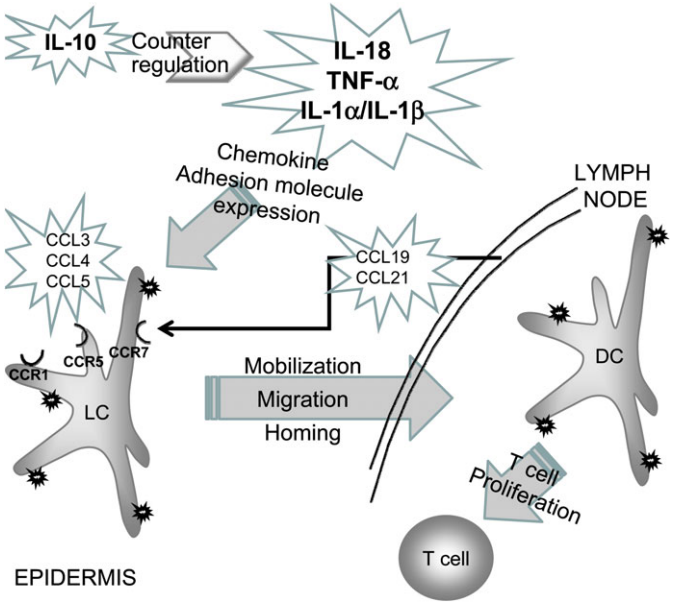

FIG. 4. A diagrammatic representation of some of the key cytokines, chemokines, and pathways involved in the initiation and directional movement of epidermal LC. Complementary, but independent, signals for mobilization are provided by IL-1β and TNF-α, with an upstream requirement for IL-18. A counterregulatory influence is provided by IL-10 acting (probably) via inhibition of TNF-α production. These cytokines collectively provoke the changes in LC that are necessary to equip them for migration out of the epidermis and transit through the skin and into the afferent lymphatics. Among the important changes is the induced expression by LC of the chemokine receptor C-C chemokine receptor type 7, which confers responsiveness to chemotactic gradients that guide the directed movement of LC toward, and localization within, skin-draining lymph nodes.

understanding that interaction with, and activation of, cutaneous DC will be a requirement for effective sensitization, and an assumption that such interactions can be modeled effectively *in vitro*.

Before considering what progress has been made specifically in the development of DC-based methods for hazard identification, it is helpful to position such investigations in the broader context of alternative and *in vitro* approaches to skin sensitization testing. It is useful to view the acquisition of skin sensitization as representing a series of hurdles that must be negotiated successfully by an allergenic chemical. These are as follows: (1) the need to gain access to the viable epidermis via the stratum corneum; (2) the ability to form stable associations with protein thereby creating an immunogenic complex (i.e., skin-sensitizing chemicals must be inherently protein reactive or they must be metabolized or otherwise transformed to a protein-reactive species in the skin); (3) the requirement for the activation, mobilization, and maturation of LC, dermal DC, or both, together with the induction or upregulation of the cytokines and chemokines necessary to sustain these processes; and finally, (4) the activation and clonal expansion of allergen-responsive T lymphocytes (Basketter and Kimber, 2009; Dearman and Kimber, 2003; Jowsey *et al.*, 2006). These key steps are illustrated diagrammatically in Figure 5 where beside each is shown the main strategies that have been adopted to address the ability of test chemicals to fulfill this requirement for sensitization.

A description of such strategies, other than those that focus on the use of DC and DC-like cells, is beyond the scope of this article. Nevertheless, it is the case that in some of the areas that we will not consider here there has been, and will continue to be, important progress. These include attempts to develop and refine further (quantitative) structure-activity relationships for skin-sensitizing chemicals (Aptula *et al.*, 2005; Cronin and Basketter, 1994; Patlewicz *et al.*, 2007), the design of peptide reactivity assays for the identification of skin-sensitizing chemicals as a function of their electrophilicity and ability to associate with proteins and peptides (Gerberick *et al.*, 2004b, 2008, 2009; Natsch *et al.*, 2007), and evaluation of changes induced by skin-sensitizing chemicals in keratinocytes (Corsini *et al.*, 2009; Vandebriel *et al.*, 2010) or a keratinocyte-based reporter cell line (Emter *et al.*, 2010).

There are also some general points that need to be made here about the challenges of developing robust and reliable alternative methods for the assessment of skin sensitization.

The first is that the immune system is complex and as a consequence is exceptionally difficult to model *in vitro* in a way that retains functional integrity. An animal, in effect, provides an integrated biological model that combines in an orchestrated fashion all the relevant immunobiological processes and events in a way that sustains an immune response. Replicating that, or even parts of that, *in vitro* from the component parts is a significant scientific and technical challenge. Second is the fact that accurate hazard identification is only the first step in the risk assessment process. What is required also is an appreciation of relative potency. This is particularly true in the case of contact allergens that as a class we believe vary by up to five orders of magnitude in their relative skin-sensitizing potential. The difficulty is that it is not yet known which of the key steps required for the acquisition of sensitization, either alone or acting in concert, most accurately reflect the relative potency of a contact allergen. Third is that a substantial proportion of contact allergens (possibly 30%) will require activation in the skin to a protein-reactive species, and this demands that *in vitro* methods should incorporate sufficient metabolic competency for this purpose. Finally, there is the purely technical, but nevertheless substantial, challenge of developing methods for introducing lipophilic chemicals to aqueous cell culture systems (Kimber *et al.*, 2010).

Against the backcloth of these challenges, the use of DC and DC-like cells as the basis for *in vitro* identification of skin-sensitizing hazards continues to be prominent among the alternative approaches being explored. Enthusiasm for this strategy can arguably be traced back to a seminal article by Alexander Enk and Stephen Katz in which they described changes in cytokine gene expression elicited in mouse skin

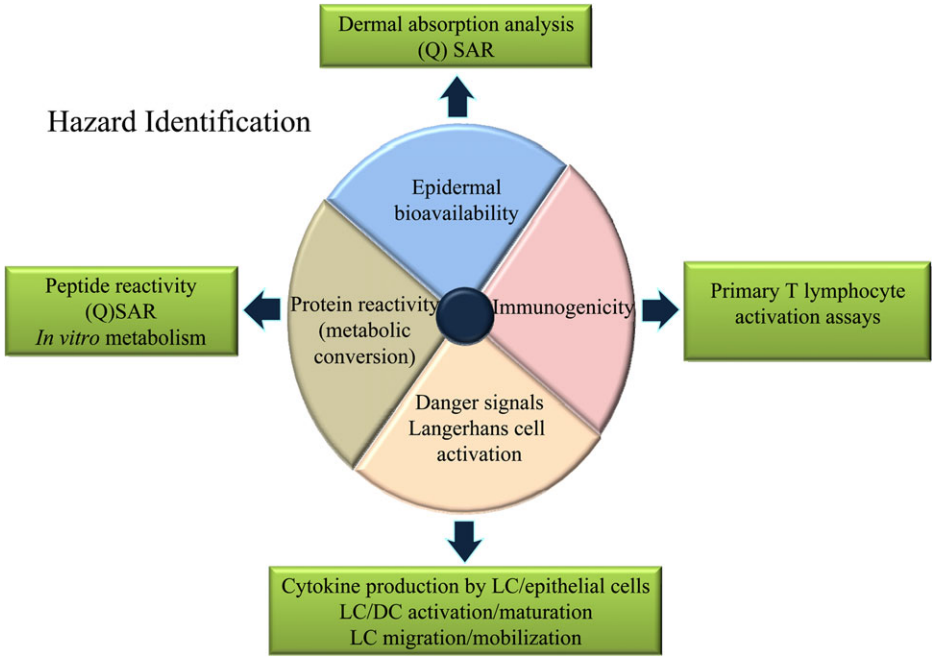

FIG. 5. A representation (in the inner circle) of the hurdles that must be negotiated successfully by a chemical if skin sensitization is to be acquired. These are (1) the need to gain access to the viable epidermis; (2) the need to form stable associations with protein and the formation of an immunogenic hapten-protein conjugate; (3) the requirement for the activation, mobilization, and migration of one or more populations of cutaneous DC and an associated need for relevant ''danger signals'' to support these processes; and (4) the activation and clonal expansion of antigen-responsive T lymphocytes. Alongside each of those requirements are identified associated strategies for alternative test methods that are being explored.

following topical exposure to contact allergens (Enk and Katz, 1992). Among the changes they observed was a rapid increase in the expression of messenger RNA (mRNA) for IL-1β; the interest being that such effects were seen with contact allergens, but not irritants, and that in mouse epidermis IL-1β is produced exclusively by LC (Enk and Katz, 1992; Enk *et al.*, 1993). This, when combined with an understanding that IL-1β is essential for LC migration and the development of skin sensitization (Cumberbatch *et al.*, 1997; Enk *et al.*, 1993), made for a fascinating story in which contact allergens rapidly provoke epidermal LC to increase their expression of IL-1β and that in turn this cytokine provides one signal for LC mobilization and sustains the acquisition of sensitization. Based on that, the thinking was that the elevation in IL-1β expression might result from a direct and selective interaction of chemical allergen with resident LC. From that inference grew the very substantial investment in the investigation of DC-based assays for skin sensitization testing. The potential value of this strategy was endorsed by a workshop convened in 1995 by European Centre for the Validation of Alternative Methods (ECVAM) (De Silva *et al.*, 1996), and since then, a variety of different cells and different endpoints have been investigated.

Progress with the design, development, evaluation, and application of DC-based assays has been reviewed extensively, and the articles cited will provide a more detailed survey than is possible here (Casati *et al.*, 2005; Galvao dos Santos *et al.*, 2009; Kimber *et al.*, 1999a,c, 2001b, 2004; Ryan *et al.*, 2001, 2005; Vandebriel and Van Loveren, 2010).

A major obstacle that had to be addressed was the availability of LC and DC. These cells are found in only very small numbers in skin and lymphoid tissue and blood, and their isolation from those sources is demanding technically. Moreover, it is clear that the isolation and manipulation of LC and DC can have a very significant impact on their phenotype and function. Despite these difficulties, freshly isolated LC have been used. Fortunately, however, there have been available other strategies for obtaining DC cells, at various stages of differentiation, by expansion of progenitor cells from blood, cord blood, or bone marrow using culture in appropriate cytokine cocktails (Bender *et al.*, 1996; Caux *et al.*, 1992; Inaba *et al.*, 1992; Lenz *et al.*, 1993; Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994).

One of the first descriptions of the use of DC from human peripheral blood for the identification of contact allergens was by Reutter *et al.* (1997) who reported that skin-sensitizing chemicals, but not sodium lauryl sulfate (SLS; a skin irritant), caused an increase in mRNA for IL-1β. Similar observations were made in one of our laboratories when it was found that several skin-sensitizing chemicals were able to provoke increases in IL-1β mRNA expression by cultured human DC. However, such responses were shown not to be consistent between subjects with cells from only approximately half the donors showing elevated IL-1β, a phenotype that appeared to

be stable with time. In agreement with the studies of Reutter *et al*. (1997), even DC derived from donors with a "responder" phenotype failed to respond to SLS (Pichowski *et al*., 2000, 2001). The conclusion drawn at that time was that such donor variability was incompatible with the further development of this approach as a method for routine testing. Of concern also was the fact that even when DC derived from a responder phenotype were used, the dynamic range of allergen-induced changes was very modest (approximately two- to threefold). Despite what at that time were seen as important limitations in the context of test development, the data were intriguing insofar as they appeared to confirm that (at least some) strong contact allergens had the potential *in vitro* to cause changes in cultured DC comparable with those that were known to be provoked in LC during skin sensitization *in vivo*. For those reasons and with an increased impetus to develop alternative methods for skin sensitization testing, investment in this area continued and continues still.

There have been a number of new developments, not least of which has been the identification of continuous cell lines that share some properties with DC (DC-like cells). Among the lines that have found greatest favor are the U937 (a human histiocytic lymphoma cell line) (Python *et al*., 2007), THP-1 (a human monocytic leukemia cell line) (An *et al*., 2009; Ashikaga *et al*., 2002, 2010; Yoshida *et al*., 2003), and MUTZ-3 (a human acute myeloid leukemia cell line) (Azam *et al*., 2006; Ouwehand *et al*., 2010; Sakaguchi *et al*., 2006). In developing assays based on these cells, much of the attention has focused on allergen-induced changes in cytokine (IL-1β, IL-6, IL-8 [CXCL8], IL-10, IL-12, IL-15, IL-18, and TNF-α) production and/or altered expression of membrane phenotypic markers (often CD54, CD80, CD86, and major histocompatibility complex [MHC] class II, and less commonly markers of LC such as E-cadherin and Langerin) (Galvao dos Santos *et al*., 2009). Recently, there has been growing interest in chemokines other than CXCL8 (IL-8, a commonly used readout for DC assays), but little information is yet available. In addition to those listed above, attempts have been made to use gene expression profiling to identify other biomarkers that might provide for an improved performance (Arkusz *et al*., 2010; Gildea *et al*., 2006; Pennie and Kimber, 2002; Python *et al*., 2009; Ryan *et al*., 2004; Schoeters *et al*., 2005; Szameit *et al*., 2009). Such analyses have met with variable success, but some interesting leads have emerged.

The interesting question is how useful are these methods as tools for predicting skin-sensitizing potential. For some approaches, and in some hands, respectable (and occasionally impressive) levels of sensitivity and selectivity are claimed, and on that basis, certain test configurations using THP-1 cells and U937 cells are currently being subjected to a formal validation. This is promising. However, it is important to recognize that in other instances, the experience has been that responses to contact allergens by DC cells or DC-like cells can be somewhat variable and inconsistent. Even if appropriate levels of reliability and reproducibility can be achieved by strict adherence to a standard protocol, there remain the issues alluded to previously to be considered, such as the following: (1) achieving the sensitivity required to detect weak allergens, (2) commonly an absence of appropriate metabolic competency, and (3) the inability (currently) to derive estimates of relative potency from DC responses. No doubt, with time, some of these problems can be solved, and with that in mind, it is appropriate to consider briefly what is on the horizon with respect to DC-based assays for skin sensitization.

One of the considerations that has intrigued these authors is the molecular basis for the recognition by DC of chemical allergens. If, as is frequently claimed, DC are able to "distinguish" between chemical allergens and nonsensitizing chemicals, including skin irritants, then it is legitimate to ask how this is achieved at a molecular level. One interesting possibility that has not, to our knowledge at least, been formally tested is that the allergen-induced responses by cultured DC are simply a reflection of the ability of electrophilic skin-sensitizing chemicals to cross-link membrane proteins displayed at the cell surface.

An alternative explanation might be that chemical allergens have a selective potential to trigger in DC, and other cell types, specific signaling pathways that drive the observed phenotypic changes. Of particular interest is the nuclear factor-erythroid 2–related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1)-antioxidant response element (ARE) pathway (Natsch, 2010; Natsch and Emter, 2008). Keap1 is a sensor protein that possesses cysteine (Cys) residues that are highly reactive with at least many haptens. In steady-state conditions, Keap1 targets, and is associated with, the transcription factor Nrf2. However, when there is covalent modification of the Cys residues by electrophilic chemical allergens, Keap1 dissociates from Nrf2, which then accumulates in the nucleus triggering transcriptional activation of genes having an ARE in their promoter regions (Ade *et al*., 2009; Megherbi *et al*., 2009; Natsch, 2010). These are exciting observations with respect to how chemical allergens are sensed, but there are additional implications that flow from these investigations.

Of particular interest is the fact that in old Nrf2 knockout mice, allergen-induced induction of the cytokine interferon-γ (IFN-γ) was inhibited, but not the production of IL-4 (Kim *et al*., 2008). The differential production of these cytokines is of considerable importance in determining the selectivity of adaptive immune responses with respect to the balance between different functional subpopulations of T helper (Th) cells (Th1 and Th2 cells). This will be considered in greater detail later in this article, but the key consideration here is that IFN-γ promotes the development of Th1 cells that are associated with skin sensitization. In contrast, IL-4 drives Th2 cell responses that in contrast are associated with sensitization of the respiratory tract (Kimber and Dearman, 1997a,b). These associations led Natsch (2010) to speculate that the covalent modification of Cys residues on Keap1 by

Cys-reactive chemical allergens will stimulate, among other changes, the production of IFN-γ that will promote Th1 cells and the acquisition of skin sensitization. The corollary is that those chemical allergens that preferentially or exclusively bind with Lys residues rather than Cys will stimulate a pathway other than Keap1/Nrf2 (as yet unknown) that will stimulate IL-4 production and Th2-type responses favoring sensitization of the respiratory tract. This is a fascinating speculation that demands further research with perhaps a particular focus on the relevance of the Keap1/Nrf2 pathway for the activation of cultured DC *in vitro*, and for the mobilization, migration, and maturation of LC and DC *in vivo*.

Another aspect of DC activation and the development of skin sensitization that may be relevant for cell-based assay systems is the requirement for "danger signals." The term danger signal was coined to describe a second signal that is required for full deployment of an adaptive immune response (Matzinger, 1998). In effect, encounter with a foreign antigen must be accompanied by a certain level of inflammation, local trauma, or cellular damage. The purpose presumably is to prevent an unnecessary and inappropriate immune response being launched when there is no threat (as signaled by local damage or trauma) of the type expected to be associated with an important incursion by antigen. Signs of damage are described collectively as pathogen-associated molecular patterns, and these are recognized via pathogen recognition receptors (Oppenhein *et al*., 2007; Vance *et al*., 2009). Of these, the most thoroughly characterized are the Toll-like receptors (TLR), of which there are many. Our view is that the need for danger signals applies also to the elicitation of immune responses to chemical allergens and the development of skin sensitization (Kimber *et al*., 2002a; McFadden and Basketter, 2000). Certainly, there is evidence now that ligation of TLR can play an important role in the development of skin sensitization. Martin and colleagues demonstrated recently that TLR4 (the membrane receptor for bacterial lipopolysaccharride) is essential for the acquisition of sensitization in mice lacking IL-12 signaling (Martin, 2010; Martin and Jakob, 2008; Martin *et al*., 2008). In addition, it would appear that endogenous ligands (including heat shock proteins Hsp27 and Hsp70) also have an important role to play (Freudenberg *et al*., 2009; Yusuf *et al*., 2009).

Perhaps, the most stark demonstration of the relevance of TLR-mediated danger signaling for skin sensitization has come from the recent and very elegant investigation of the role played by TLR4 in nickel allergy (Schmidt *et al*., 2010). Nickel is a very common cause of ACD in North America and Europe. The conundrum for experimental dermatologists and toxicologists has been that it has proven very difficult, and often impossible, to stimulate sensitization to nickel salts in mice and other laboratory animals. In the local lymph node assay (LLNA) and in other tests for skin sensitization, nickel is regarded (correctly) as a false negative. The paradox has now been solved, and the immunobiological basis for this species difference has been explained. It was shown that the nickel ions can directly trigger activation of human TLR4. This activation is dependent upon the presence of nonconserved histidines at positions 456 and 458 of the receptor; these residues being found on human TLR4, but not on the equivalent receptor in mice (Schmidt *et al*., 2010). The significance of this discovery is that in humans, but not in mice, Ni^{2+} ions can induce skin sensitization because, via activation of TLR4, it is possible to provoke the inflammatory signals that are necessary to initiate and sustain an adaptive immune response. Furthermore, transgenic expression of human TLR4 in TLR4-deficient mice was shown to enable effective sensitization to nickel (Schmidt *et al*., 2010).

In effect, danger signals form a bridge between the innate and adaptive immune systems, and although other cells are affected by sensing of these signals, it is likely that in the context of skin sensitization at least that cutaneous DC are key targets. Certainly, it has been shown that some TLR ligands cause the activation of DC *in vitro* and that specific TLR ligands may also imprint on DC-polarizing capability with respect to driving selective Th1 or Th2-type responses (Dearman *et al*., 2009b; Dzopalic *et al*., 2010; Sen *et al*., 2010; Zanoni and Granucci, 2010). Of particular relevance is the fact that in some circumstances, chemical allergens and specific TLR ligands may have synergistic effects on the activation of DC (Dearman *et al*., 2008b).

Related to the need for appropriate danger signals and in the context of the conduct and performance of DC-based assays, it may be relevant that it is not uncommon to find some association between loss of cell viability and induced changes in DC phenotype. That is, the concentrations of chemical allergen that are found to provoke changes in cytokine production or the expression of membrane markers are frequently close to those at which a reduction in viability is noted. One speculation is that, in some instances, trauma caused by exposure of cells to the test chemical is itself sufficient to stimulate the observed phenotypic changes. The argument would be, therefore, that other causes of cell damage may also be associated with effects that are attributed to treatment with chemical allergens. More likely, however, is the explanation that a certain level of cellular trauma will be associated with the release of endogenous danger signals that will act in synergy with chemicals to induce or augment DC activation. Certainly, the sensing of necrosis by DC has been linked to their immunological function (Sancho *et al*., 2009).

Irrespective of the mechanisms through which endogenous and exogenous danger signals impact on DC activation, it would appear that harnessing and exploiting their biological properties may be a fruitful way of enhancing the performance of cell-based assays for sensitization. In this context, it can of course be argued that mixed culture systems, such as, for instance, coculture of DC and keratinocytes, might be a more appropriate model system providing greater opportunity for the

elaboration of factors that support DC activation (Schreiner *et al*., 2007; Wanner *et al*., 2010).

Before leaving LC, DC, and novel predictive tests for skin sensitization, there are two other approaches that warrant a mention.

The first is that there has been for some years an investment in the elaboration of 3D models of skin that can be used for toxicity testing and other purposes. With respect to *in vitro* test development, this represents a potentially attractive approach, not least because such models commonly comprise cells grown on membranes at the air/liquid interface and this facilitates delivery of lipophilic chemicals. There are commercially available models of reconstructed human epidermis, but currently none of these incorporates either LC or DC. Nevertheless, 3D skin models that incorporate DC are now being described (Uchino *et al*., 2009).

The other consideration is the opportunity of measuring *in vitro* the impact of chemical allergens on the mobilization and migration of DC-like cells. There has recently been described a transwell assay based upon the selective migration of MUTZ-3 LC-like cells toward the chemokine CXCL12 following exposure to skin-sensitizing chemicals (Ouwehand *et al*., 2010). Experience to date is limited to a relatively small number of test chemicals, but it is possible that this approach may bear fruit. What is not clear, however, is whether this experimental system would be sufficiently tractable for use as a routine predictive assay.

In conclusion, there has been a really substantial global investment in the development of LC/DC-based assays. Progress has been made, but it is arguable whether the degree of progress is commensurate with the scale of investment during the last decade or more. Certainly, it is possible to signal some achievements. These include the ability to expand in culture populations of DC, of various phenotypes, from progenitors, and the availability of DC-like cell lines, a more detailed understanding of the changes that are elicited by exposure of cultured DC to chemical allergens, and a growing appreciation of the impact that cofactors may have on the activation of DC. In addition, it must be acknowledged that currently two DC assays for skin sensitization (based on the use of THP-1 and U937 cells) are being considered within a formal validation process.

However, many challenges remain; some of them are significant. All cell-based assays are associated with difficulties of maintaining phenotype and delivery of test chemicals to aqueous culture systems. Moreover, there is not yet a consensus regarding which cell system is most appropriate or the readouts that provide the most sensitive and selective alerts for skin-sensitizing activity. There is a need also to determine whether a single cell type or cell line is sufficient for the purposes of hazard identification or if a coculture model is required. It should be possible to address and resolve at least most of these issues. But, even if there emerges a DC-based assay that provides reliable information about skin sensitization hazards, it is very unlikely that it will, in isolation, provide a complete replacement for currently available methods such as the LLNA. The thinking currently is that the solution to prediction of skin-sensitizing activity without recourse to experimental animals will demand the careful integration of two or more different *in vitro*/*in silico* "tests" that presumably reflect different aspects of the sensitization process.

Even then, there will be the need to provide information regarding relative skin sensitization potency rather than solely hazard identification. As will be described later, there is good reason to believe that the LLNA is able to deliver this; the challenge is to derive equivalent information from an *in vitro* test or battery of tests. What is required is the identification of induced changes or markers that have not only a causal relationship with the acquisition of skin sensitization but also a quantitative correlation. In the context of the foregoing discussion, it is, therefore, relevant to question whether changes provoked in cultured DC by chemical allergens might be reflective of the degree to which sensitization is achieved and suitable for hazard characterization. The answer is that we simply do not know. It is clear that many events and processes represent mandatory requirements for the acquisition of sensitization, but which of these also determine sensitizing potency remains unknown. So, in the context of LC/DC function during the induction phase of skin sensitization, it is not clear whether there is a threshold requirement for mobilization and maturation or whether metrics such as (for instance) the number of cells that become mobilized, the speed of mobilization and migration, or the degree of maturation govern differences between chemical allergens with respect to sensitizing potency. Until we have a more complete understanding about the dynamics of LC and DC function in relation to skin sensitization, it may not be possible to equate the results of DC-based assays with relative potency.

It will be interesting to view future developments in this area and whether a growing appreciation of the signals and pathways that orchestrate DC activation can be exploited to develop improved test strategies.

There is one final important reflection on DC-based assay systems that is relevant in the context of developing integrated testing strategies. Interest in such strategies is based on the assumption that pooling of information about the behavior of a chemical in different assay systems will provide a more holistic view of potential sensitizing activity. The argument is that data on, for instance, protein-peptide binding and the ability to activate DC-like cells will necessarily be complementary and therefore more informative. However, in this particular example, it could be argued that the reported ability of chemical allergens to cause changes in the phenotype of DC is simply, and solely, a reflection of the ability of electrophilic chemicals to bind to cell-associated proteins nonspecifically or to bind with particular sensing proteins such as Keap1. If this is in fact the basis for chemical allergen–induced DC activation, then a case can be made that DC-based assay systems do

nothing more than provide a rather complicated method for evaluating the protein-binding potential of chemicals and that such assays add no value compared with approaches that seek to measure directly peptide binding in cell-free systems. The counterargument is, of course, that even if the activation of DC is dependent on protein binding by chemical allergens, the assessment of this within the context of a cell type known to play an important role in the acquisition of skin sensitization is of far greater relevance than are data based on association with model peptides in a test tube.

Ultimately, the relevance of LC and DC function during the induction phase of skin sensitization is to provide a mechanism for the recognition, processing, delivery, and presentation of chemical allergen to responsive T lymphocytes. Presentation takes place in peripheral lymph nodes draining the sites of skin exposure to chemical allergen and results in the activation and clonal expansion of allergen-reactive T lymphocytes. This can be regarded as the central event in skin sensitization, and approaches to hazard identification and characterization based on T lymphocyte responses are considered next.

T LYMPHOCYTES AND SKIN SENSITIZATION

The role of T lymphocytes in the induction of skin sensitization and elicitation of ACD is an enormous and an enormously complicated subject. For the purposes of this article, attention will focus exclusively on the exploitation of T lymphocyte responses for hazard identification and hazard characterization of skin-sensitizing chemicals. Within this framework, we will consider briefly the murine LLNA, a method based on characterization of T lymphocyte activation and characterization in skin-draining lymph nodes, and also attempts that have been made to develop alternative approaches based upon measurement of T lymphocyte priming *in vitro*.

The LLNA was first developed toward the end of the 1980's as an alternative to guinea pig tests (guinea pig maximization test, Magnusson and Kligman, 1969, and the occluded patch test of Buehler, 1965) for the identification of contact allergens (Kimber and Weisenberger, 1989; Kimber *et al*., 1986, 1989). There is a burgeoning literature associated with the LLNA, and many review articles and historical surveys are available (Basketter *et al*., 2002; Cockshott *et al*., 2006; Dearman *et al*., 1999; Gerberick *et al*., 2000; Kimber and Basketter, 1992: Kimber *et al*., 1994, 2002b; McGarry, 2007).

The assay is predicated on an understanding that the acquisition of skin sensitization is dependent upon the initiation of an immune response in lymph nodes draining the site of exposure to a contact allergen. The argument was that skin-sensitizing chemicals will cause an activation of draining lymph nodes and can be identified on that basis. Lymph node activation is triggered by the arrival of antigen borne by DC migrating from the skin and is associated with increases in lymph node weight and cellularity and with the stimulation of T lymphocyte proliferation. In preliminary investigations, a variety of markers of lymph node activation were considered, but it became apparent that the most sensitive and most selective metric was lymph node cell turnover measured as a function of the incorporation of [^{3}H] thymidine (^{3}H-TdR). In fact, it was found that improved sensitivity could be achieved if the isotope was administered *in situ* following iv administration of ^{3}H-TdR (Kimber *et al*., 1989). It is this approach that was developed and eventually validated formally.

Detailed accounts of the protocol used for standard versions of the LLNA are available elsewhere (Gerberick *et al*., 1992; Kimber and Basketter, 1992; Kimber and Dearman, 2010). Very briefly, the assay is conducted as follows. Groups of mice (CBA strain) receive topical application on the dorsum of both ears of various concentrations of the test material (in an appropriate vehicle) or an equal volume of the relevant vehicle alone. The same treatment is repeated daily for 3 consecutive days. Five days following the initiation of exposure, animals are injected iv with a source of ^{3}H-TdR. Mice are sacrificed 5 h later, and the draining (auricular) lymph nodes are excised, pooled for each experimental group (or for each mouse), and processed for β scintillation counting. Data are recorded as disintegrations per minute from which a stimulation index (SI) for each dose group is derived relative to vehicle control values. Skin-sensitizing chemicals are defined as those that, at one or more test concentrations, elicit a threefold or greater increase in lymph node cells (LNC) proliferation compared with concurrent vehicle controls (an SI of three or more) (Kimber *et al*., 2002b). Although the very abbreviated protocol described above was the version of the LLNA that was developed originally, and validated formally, there have been a variety of modifications proposed; some being more radical than others (Basketter *et al*., 2008; Ehling *et al*., 2005; Gerberick *et al*., 1992; Ikarashi *et al*., 1993; Ladics *et al*., 1995; Sailstad *et al*., 1995; Takeyoshi *et al*., 2001; Ulrich *et al*., 2001; Yamano *et al*., 2005; Yamashita *et al*., 2005). The fact that modified protocols have been described is acknowledged, but their limitations or merits compared with the standard assay are not explored further here.

Following its original description, the LLNA was evaluated rigorously in both national and international interlaboratory collaborative trials (Basketter *et al*., 1991; Kimber and Basketter, 1992; Kimber *et al*., 1991, 1995, 1998b; Loveless *et al*., 1996; Scholes *et al*., 1992). Moreover, there were conducted extensive comparisons between the LLNA and data available from guinea pig tests and from experience of human skin sensitization (Basketter and Scholes, 1992; Basketter *et al*., 1991, 1992, 1993, 1994; Edwards *et al*., 1994; Kimber *et al*., 1994; Ryan *et al*., 2000). During the course of those evaluations and comparisons and as the subject of other investigations (Kimber *et al*., 2002b), experience was gained with a wide variety of chemicals.

A schema illustrating the basic standard protocol for the LLNA is provided in Figure 6, together with a diagrammatic

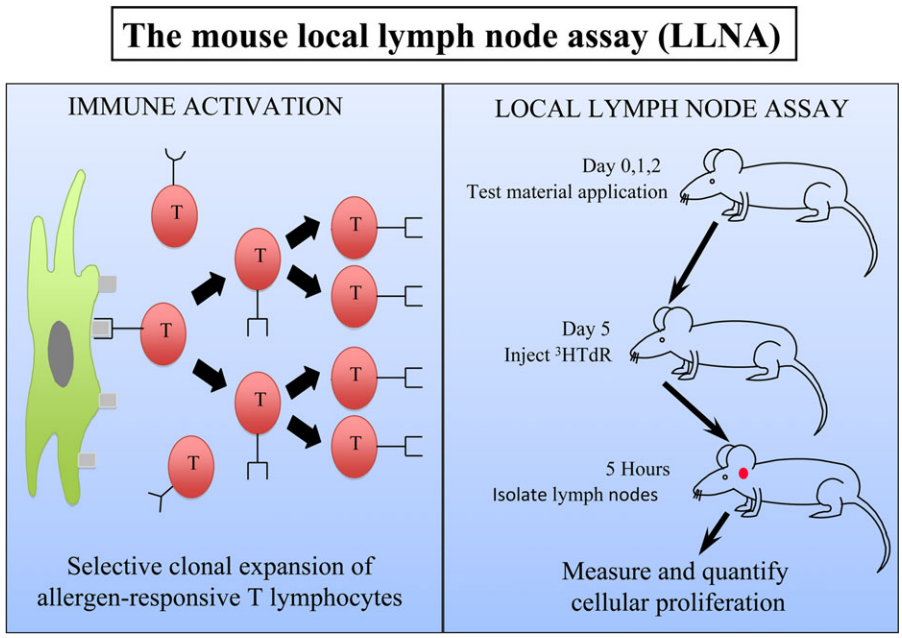

FIG. 6. The murine LLNA. Left box. The immune activation and clonal expansion of allergen-reactive T lymphocytes in skin-draining lymph nodes; the immunological event upon which the LLNA is based. Right box. The basic LLNA protocol. Groups of CBA strain mice receive a topical application on the dorsum of both ears of various concentrations of the test chemical or an equal volume of the vehicle alone. Treatment is repeated daily for 3 consecutive days. Five days following the initiation of exposure, mice are injected (iv) with a source of ^{3}H-TdR. Mice are sacrificed 5 h later and the draining auricular lymph nodes excised and processed for β scintillation counting.

representation of the aspects of the immune response to skin-sensitizing chemicals (T lymphocyte activation and clonal expansion) upon which the assay is based.

Collectively, the studies summarized above suggested that the performance of the LLNA was such that it might serve as a viable alternative to guinea pig tests (the guinea pig maximization test and the occluded patch test of Buehler) (Basketter *et al.*, 1996; Chamberlain and Basketter, 1996; Gerberick *et al.*, 2000). The next step was that in 1998, the LLNA was submitted for consideration by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). After exhaustive scrutiny by an expert peer review Panel, the conclusion drawn was that, compared with other predictive tests for skin sensitization (guinea pig assays), the LLNA offered important animal welfare benefits (a need for fewer animals and a reduction in trauma and discomfort). In addition, the Panel recommended that, subject to the introduction of some procedural amendments, the LLNA could be used as a stand-alone method for the predictive assessment of skin-sensitizing activity (Dean *et al.*, 2001; Haneke *et al.*, 2001; NIH, 1999; Sailstad *et al.*, 2001). In the wake of the recommendations from ICCVAM, the assay was endorsed also by the ECVAM (Balls and Hellsten, 2000). The LLNA was subsequently (April 2002) incorporated into a new Test Guideline (no. 429; Skin Sensitization: Local Lymph Node Assay) by the Organization for Economic Cooperation and Development.

Since formal validation, the LLNA has been used increasingly. For the most part, experience has borne out the value of the assay for hazard identification. It has been our view that the LLNA displays an overall level of accuracy of approximately 90%, a view that is line with conclusions drawn by the ICCVAM Panel. This level of performance is comparable with estimates of the accuracy of the guinea pig maximization test (Basketter and Kimber, 2010; Dean *et al.*, 2001) and is indeed consistent with test methods that are available for the identification of a wide range of hazards. Predictive tests are not perfect, and there will always be a need for judicious interpretation based upon an appreciation of the strengths and limitations of a method (Basketter *et al.*, 2010). It is inevitable, therefore, that there have arisen issues relating to performance of the LLNA.

One concern was that the LLNA is not able to identify as positive nickel salts (Kimber *et al.*, 1994), the same being the case with guinea pig tests. This was galling, not because nickel is a potent contact allergen (which it appears not to be) but rather because in humans it is a very common cause of ACD. It is now clear that the failure of nickel salts to elicit positive responses in the LLNA is attributable to species selectivity at the level of danger signal generation (Schmidt *et al.*, 2010). It has been shown that nickel ions can directly trigger activation of TLR4 in humans, but not in mice. Activation by Ni^{2+} of human TLR4 requires the presence of two nonconserved histidine residues that are present on the human receptor, but

not mouse TLR4 (Schmidt *et al.*, 2010). Nickel is something of a special case, and the available evidence is that with other metal allergens, the performance of the LLNA is satisfactory. This view is supported by a systematic evaluation of the behavior of metals in the LLNA (Basketter *et al.*, 1999a). In that survey, 13 metal salts were investigated, of which 8 were classified as contact allergens, with the remaining 5 being nonsensitizers. With the exception of nickel chloride, the known metal allergens were all shown to elicit positive responses in the LLNA. Of the 5 nonsensitizers, 4 failed to induce LLNA responses (Basketter *et al.*, 1999a).

The other general consideration is the possibility that some classes of chemicals may provoke false-positive reactions in the LLNA. Inevitably, all test methods will display some level of false-negative and false-positive responses, and the same is not unexpected with the LLNA. One focus of attention has been the behavior of nonsensitizing skin irritants in the assay, the concern being that local irritation or inflammation, even in the absence of an allergenic signal, may be sufficient to cause a positive response. Certainly, that has been the experience with SLS (a nonsensitizing chemical that causes skin irritation) that has been found to induce relatively modest, but nevertheless positive, responses in the LLNA (Kimber *et al.*, 1994; Loveless *et al.*, 1996; Montelius *et al.*, 1994). The reason why SLS provokes lymph node activation is not known, but the available evidence suggests that skin irritation *per se* does not necessarily elicit a positive response in the LLNA (Basketter *et al.*, 1998).

In practice, skin irritation is not an important confounder in the LLNA (Basketter *et al.*, 1998). Nevertheless, there is one approach that has been developed, for use as an adjunct to the standard LLNA, that appears to permit discrimination between lymph node responses resulting from allergen exposure and immune activation from what might be viewed as nonspecific or irritation-induced responses. This supplementary procedure is based upon enumeration of the frequency of $B220^+$ B lymphocytes in draining lymph nodes. In response to chemical allergens, but not other chemicals, there is a significant increase in B-cell numbers (indicative of the initiation of a cutaneous immune response). This procedure has proven to be of value in confirming the sensitizing potential of chemicals in instances where (for whatever reason) there is reason to doubt a positive response in the LLNA (Betts *et al.*, 2007; Gerberick *et al.*, 2002).

However, it is clear that in some cases, the LLNA, in common with guinea pig assays (Basketter and Kimber, 2010), may be associated with true false positives. Nevertheless, these are no more prevalent than one would expect in the wake of extensive application of the method with a wide range of chemistries. What will be interesting and instructive is to resolve at a molecular level why some nonsensitizing chemicals when applied topically are able to provoke changes in draining lymph nodes that resemble immune activation (Basketter *et al.*, 2009a,b; Kreiling *et al.*, 2008).

Before leaving the LLNA in relation to hazard identification, two other issues are worthy of a brief mention.

The first is that there has been interest recently in exploring whether in certain circumstances a "cut down" or "reduced" LLNA could be used to achieve further reductions in the number of animals required for hazard identification. The reasoning was that in some circumstances where the emphasis is on screening, rather than on developing a detailed risk assessment, it might be possible to deploy a different configuration of the LLNA employing only a single high-dose group and a concurrent vehicle control group. Experience to date suggests that this approach can distinguish between sensitizing and nonsensitizing chemicals (Kimber *et al.*, 2006). There has been some interest also in exploring whether the number of mice within treatment groups can be reduced without a detrimental impact on the performance of the LLNA (Ryan *et al.*, 2008).

Second is the fact that the accumulated experience with the LLNA has provided an opportunity to develop and curate extensive data sets that can be, and are being, used as a basis for evaluating the sensitivity, selectivity, and overall performance of putative alternative test methods (Gerberick *et al.*, 2004a, 2005; Kern *et al.*, 2010).

It is clear therefore that the LLNA, based upon the measurement of proliferative responses induced in regional lymph nodes, provides a reliable method for identifying skin sensitization hazards. However, hazard identification represents only the first step in a toxicological evaluation. Accurate risk assessments and the development of risk management strategies demand an appreciation of relative potency. This is of course a requirement in all areas of toxicology but is of particular importance in chemical allergy because it appears that contact allergens vary by up to five orders of magnitude with respect to their relative skin sensitization potency. As discussed previously, characterization of relative potency represents one of the most significant challenges in the design of alternative methods for skin sensitization testing.

At present, the preferred approach for measuring relative skin-sensitizing potency is based upon evaluation of dose-response relationships in the LLNA. Although it is possible in some instances to infer estimates of relative potency from well-conducted guinea pig assays, this is not always the case, and such assays are not normally configured to allow characterization of dose metrics for the induction of sensitization.

The value of the LLNA in this context is that there are sound immunological reasons to believe that the magnitude and vigor of T lymphocyte proliferative responses induced by chemical allergens in regional lymph nodes will determine the extent to which sensitization is acquired. Simply, the more vigorous the proliferative response the greater will be the expansion of antigen-reactive memory/effector T lymphocytes (Friedmann, 1990). This expectation is borne out by experimentation (Kimber and Dearman, 1991; Kimber *et al.*, 1999b). Therefore, T lymphocyte proliferation in draining lymph nodes bears not

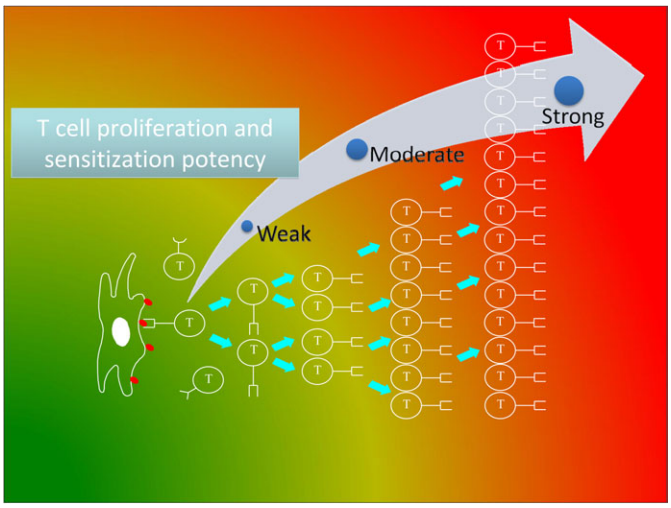

FIG. 7. A pictorial representation of the relationship between the vigor of allergen-induced T lymphocyte proliferation, the relative potency of skin sensitization, and opportunities for the improved classification of contact allergens.

only a causal link with the acquisition of skin sensitization but also a quantitative relationship.

Attention focused on how best to exploit the LLNA for the purposes of hazard characterization and the measurement of potency (Kimber and Basketter, 1997; Kimber *et al.*, 2001a). The approach adopted was to derive from dose-responses in the LLNA an EC3 value; this being the amount of chemical required to elicit an SI of three in the assay (a threefold increase in proliferative activity compared with concurrent vehicle control values). The units for the EC value can be the absolute amount of chemical (measured as percent wt/vol or as a molar value) or as the amount of chemical per unit area of skin (the latter being the important exposure metric for the development of skin sensitization).

Consideration was given to the most appropriate method for deriving EC3 values (Basketter *et al.*, 1999b). The decision reached was that linear interpolation of values either side of an SI of three provides the most convenient and most robust approach (Basketter *et al.*, 1999b).

Extensive experience has revealed that the EC3 value is a reliable and reproducible measure of activity in the LLNA (Basketter *et al.*, 2007a,b; Kimber *et al.*, 2002b; Warbrick *et al.*, 1999). Importantly, it has been established that EC3 values derived from the LLNA correlate closely with clinical judgments of skin-sensitizing potency among humans. In two separate investigations, there was found to be a close association between clinical assessments of potency and EC3 values, with those chemicals considered to be the most potent contact allergens having the lowest EC3 values in the LLNA (Basketter *et al.*, 2000; Gerberick *et al.*, 2001). There has been reported also a correlation between potency as measured in the LLNA and human skin sensitization thresholds (Basketter *et al.*, 2005b; Schneider and Akkam, 2004).

If one accepts that EC3 values provide a relevant measure of potency (Basketter *et al.*, 2007a,b), then it is not unreasonable to propose that it should prove possible to categorize or classify contact allergens according to their relative skin-sensitizing activity. This work is still in progress, and there is no real consensus yet regarding how many potency classifications would be most appropriate. Nevertheless, this is an issue worth pursuing because, for the purposes of risk management, an appreciation of potency would be of far greater value than a simple binary separation between sensitizing and nonsensitizing chemicals (Basketter *et al.*, 2005a; Kimber *et al.*, 2003; Loveless *et al.*, 2010).

The principle of relating the vigor of T lymphocyte proliferation to relative skin-sensitizing potency and the categorization of contact allergens is illustrated diagrammatically in Figure 7.

In conclusion, therefore, the LLNA demonstrates that it is possible to identify and characterize skin-sensitizing chemicals without the requirement (as is the case with guinea pig tests) to elicit in animals contact allergic reactions. This is achieved by exploiting the fact that the acquisition of skin sensitization is dependent upon the activation of T lymphocytes and that T lymphocyte responses correlate quantitatively with sensitization thresholds and the extent of sensitization achieved. It is relevant, therefore, to examine whether it is possible to use the measurement of T lymphocyte responses as the basis for *in vitro* testing. Such a strategy appears both legitimate and attractive but in practice is associated with a number of challenges, the most significant of which is the level of sensitivity required to monitor *in vitro* the stimulation by chemical allergens of primary T lymphocyte responses.

Chemical allergens are unable themselves to provoke adaptive immune responses, and for this reason, they are described as haptens. In most instances, to elicit responses, chemicals must form stable associations with protein. T lymphocyte epitopes are generated through processing of these hapten-protein conjugates by DC. Such processing results in the production of chemically modified peptides that are displayed at the surface of mature DC in association with MHC gene products (MHC class I and MHC class II molecules). It is this MHC-hapten-peptide complex that is recognized by T lymphocytes with complementary T-cell receptors (TCR) (Martin, 2004; Martin *et al.*, 1992; Ortmann *et al.*, 1992). There are some exceptions. Metal ions, such as nickel, are able to form noncovalent complexes directly with MHC molecules and/or the TCR (Gamerdinger *et al.*, 2003; Lu *et al.*, 2003; Thierse *et al.*, 2005). Finally, it has been proposed also that some haptenic drugs may activate specific T lymphocytes by mechanisms independent of an association with peptides anchored within MHC molecules; the so called p-i, or peptide-independent, model (Schnyder *et al.*, 1997). It has not yet been established whether, and to what extent, the p-i concept is relevant for organic contact allergens.

Whatever the mechanisms that result in the display of an immunogenic peptide and irrespective of the components of the MHC-peptide-TCR complex to which the peptide is bound, in a naive population of T lymphocytes, only a very small fraction of cells will bear complementary receptors. That is, among naive T lymphocytes, the number of cells that will become activated and proliferate in response to a specific antigenic signal will be very low.

It is possible to distill from various estimates some general metrics that serve to put this into context. It can be assumed that in a normal adult human, there are approximately 10^{12} T lymphocytes, and it may be that among these are represented 25×10^6 different T lymphocytes with respect to TCR specificity. This would suggest that in any pool of naive T lymphocytes, cells of a particular specificity will represent only a tiny fraction of 1% ($1{:}10^5$ or $1{:}10^6$). Experience with peptide antigens in mice is in accord with this (Moon *et al.*, 2007). The message is, therefore, that even if some contact allergens are found to have somewhat larger TCR repertoires than is the norm, the measurement of primary allergen-specific T lymphocyte responses *in vitro* represents a significant technical challenge. The issue is, in effect, that there will be expected to be a low signal:noise ratio.

A second issue of potential relevance is that the stimulation threshold for antigen-induced activation of potentially responsive naive T lymphocytes is higher than that required for the provocation of responses by memory/effector cells (Inaba and Steinman, 1984). It is generally accepted that one manifestation of this is that, although some other cell types are able to present antigen to trigger secondary immune responses, DC are required for the activation of naive T lymphocytes and for the initiation of primary immune responses.

One attempt to address these issues was reported by Hauser and Katz (1988). They used hapten-modified LC to activate T lymphocytes from naive mice. However, spontaneously reactive T lymphocytes activated by culture with unmodified LC were first eliminated using bromodeoxyuridine and light. Using this strategy, antigen-specific responses by naive T lymphocytes were reported (Hauser and Katz, 1988).

In the period since then, there has been a variety of protocols used to stimulate naive T lymphocyte responses—commonly proliferative responses—to (usually) potent chemical allergens (Dai and Streilein, 1998; Dietz *et al.*, 2010; Guironnet *et al.*, 2000; Krasteva *et al.*, 1996; Moulon *et al.*, 1993; Rougier *et al.*, 1998, 2000; Rustemeyer *et al.*, 1999; Vocanson *et al.*, 2008). There are a number of variables that require consideration in developing strategies for the design of T lymphocyte assays. These include the following: (1) the phenotypic characteristics of the T lymphocytes themselves, (2) the most appropriate readouts for the measurement of allergen-driven T lymphocyte activation, and (3) the method used for delivery of the relevant antigen to T lymphocyte cultures (Martin *et al.*, 2010).

The aim of T lymphocyte–based assays for hazard identification is to examine the ability of chemicals to stimulate responses by naive cells. It is possible to distinguish between memory cells (resulting from previous immunological priming), and virgin T lymphocytes on the basis of CD45 expression. Memory cells display a truncated form of this molecule (CD45RO), whereas virgin T lymphocytes possess the higher molecular weight isoform (CD45RA). The differential expression of CD45 isoforms has been used by some investigators to verify that allergen-induced responses do indeed reflect the activation of naive T lymphocytes (Moulon *et al.*, 1993; Rougier *et al.*, 1998).

There is an interplay between various classes and subclasses of T lymphocytes in the induction, regulation, and elicitation of ACD (Cavani *et al.*, 2001; Grabbe and Schwarz, 1998: Kimber and Dearman, 2002a; Vocanson *et al.*, 2009b). One of the interesting observations that has been made in a number of experimental systems is that $CD4^+$ T lymphocytes may actually play an immunoregulatory role in ACD (Bour *et al.*, 1995; Cavani *et al.*, 1998; Gocinski and Tigelaar, 1990; Kimber and Dearman, 2002a; Vocanson *et al.*, 2009b). In this context, it is interesting that in mice, removal of all $CD4^+$ T lymphocytes has been found to potentiate responses to contact allergens (Lass *et al.*, 2008; Vocanson *et al.*, 2006, 2009a). This is consistent with our increasing understanding of the roles played by regulatory $CD4^+$ T lymphocytes (Treg cells) as important endogenous controllers of the adaptive immune response (Sakaguchi *et al.*, 2010; Shevach, 2009; Vignali, 2008; Vignali *et al.*, 2008; Workman *et al.*, 2009). Natural Treg cells are characterized by the phenotype $CD4^+$ $CD25^+$ $FoxP3^+$ (the latter being a transcriptional regulator that serves as a "master switch" for the development and function of Treg cells) (Zhang and Zhao, 2007). It has been found that elimination of Treg cells from responder populations can increase substantially the sensitivity of T lymphocyte assays for contact allergens (Vocanson *et al.*, 2008).

If T lymphocyte populations depleted of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells possibly represent the most appropriate responder population (at least currently), the next question regards the best and most sensitive readout for such assays. Most of the reports cited above have employed T-cell proliferation as the readout of choice, although the induction of cytokine expression (usually IFN-γ) has sometimes been used in tandem (Dietz *et al.*, 2010; Vocanson *et al.*, 2008). Although these endpoints are relevant and reflective of T lymphocyte activation, it may be that other markers offer improved sensitivity. Among those that have been suggested is the ligand for CD40 (CD154), a membrane determinant that is upregulated on antigen-activated T lymphocytes (Elgueta *et al.*, 2009).

One of the most critical considerations may prove to be the form in which allergen is delivered to cultures of T lymphocytes. In some experimental systems, chemical allergen has been added directly, and although this may be an

appropriate strategy for aqueous solutions of metal salts, it is (as has been discussed above) less workable with lipophilic chemicals. The other commonly used method has been to prime DC with hapten prior to addition of these cells to culture. Although this is a feasible (and in at least some instances a successful) approach, it is not possible to ensure that the process of hapten modification has not compromised in some way the functional integrity of DC with respect to antigen presentation. Also, it is possible that it is not the hapten-modified DC themselves that present antigen in culture. The suspicion is that, in some experimental systems, the DC bearing hapten in fact provide only a means of introducing the chemical allergen to culture, and presentation is actually performed by other antigen-presenting cells within the responder population.

One other option is to introduce instead hapten-protein or hapten-peptide conjugates. This appears attractive as it is possible to characterize such conjugates and use them as reagents (Aleksic *et al.*, 2007, 2009; Dietz *et al.*, 2010; Jenkinson *et al.*, 2010). Among the characteristics of conjugates that can be manipulated and measured is the molar substitution ratio. However, although this provides the option of comparing and contrasting the immunogenic properties of proteins bearing differing amounts of hapten, it is not possible in advance to identify the ideal substitution ratio for use with a particular chemical in a T lymphocyte priming assay. The other issue that warrants consideration is the fact that, although the direct addition of chemical or hapten-modified DC to culture may provide a source of trauma-derived danger signals to support optimal presentation, this might not be the case with hapten-protein conjugates that are likely to be more "benign." In the latter case, it may prove necessary to consider the addition to culture also of proinflammatory cytokines or other soluble factors (Rustemeyer *et al.*, 1999).

Even if the issue of chemical delivery can be resolved satisfactorily and there are in place appropriate protocols for preparation of optimal T lymphocyte responder populations, there is still the problem of sensitivity to tackle. One strategy that holds promise is to prime naive T lymphocytes with the relevant chemical allergen and then to restimulate subsequently with the same allergen or with (as controls) unrelated chemicals as a test of specificity. Although this is feasible, it is a complicated and relatively lengthy experimental procedure that would not lend itself readily to the needs of routine testing.

T lymphocyte–based assays are certainly attractive, and there are real signs of progress. However, as will be apparent from the foregoing discussion, there remains no shortage of technical challenges. Included among those, but not considered here, is the need (in common with other *in vitro* approaches to testing) to accommodate chemicals that require enzymatic or oxidative transformation to a sensitizing species. Many of those hurdles will no doubt be overcome, and it is possible that it may become possible in the future to assess reliably and on a routine basis the ability of chemicals, or chemical-protein conjugates, to provoke the activation of naive T lymphocytes *in vitro*.

However, even if such assays become available, it would be necessary to treat the information they supplied with some caution. It is difficult to anticipate whether the apparent failure of a chemical or conjugate to activate naive T lymphocytes *in vitro* would provide reassurance of the lack of sensitizing activity. Conversely, it is not clear whether such activation would necessarily implicate the chemical as a contact allergen. Only time will tell. But in the meantime, this remains a fertile area of research and certainly one that is deserving of continued interest and investment.

ACD is a common occupational and environmental health problem, and many hundreds of chemicals are known to cause skin sensitization. In fact, ACD is the commonest manifestation of immunotoxicity in humans. There is, however, another form of chemical allergy that is relevant in an occupational setting, sensitization of the respiratory tract.

SENSITIZATION OF THE RESPIRATORY TRACT

The ability of some chemicals to cause allergic sensitization of the respiratory tract and asthma is a significant occupational health problem, not least because it is associated with high levels of morbidity and can be fatal (Chester *et al.*, 2005; Kimber and Wilks, 1995; Newman Taylor, 1988). Moreover, this form of chemical allergy poses significant toxicological problems, not least because, as yet, there are no available validated or even widely accepted predictive test methods. The lack of standard methods for toxicological evaluation is in large part because of the absence within the scientific community of a clear consensus about the immunobiological mechanisms through which chemicals cause sensitization of the respiratory tract. This is an issue to which we will return.

It is not possible within the space available here to explore all aspects of respiratory sensitization and occupational asthma, and there are available elsewhere a number of surveys, review articles, and workshop reports that highlight the important clinical, immunological, and toxicological issues (Banks and Tarlo, 2000; Boverhof *et al.*, 2008; Briatico-Vangosa *et al.*, 1994; Holsapple *et al.*, 2006; Isola *et al.*, 2008; Kimber and Dearman, 2005; Kimber *et al.*, 1996, 2007; Liu and Wisnewski, 2003; Sastre *et al.*, 2003; Selgrade *et al.*, 1994; Tarlo and Malo, 2006, 2009).

In contrast to the situation that pertains to ACD, there are only a relatively small number of chemical classes and individual chemicals that have been unequivocally implicated as having the potential to cause allergic sensitization of the respiratory tract. Among these are the diisocyanates (Zammit-Tabona *et al.*, 1983), acid anhydrides (Bernstein *et al.*, 1982; Zeiss, 2002), some platinum salts (Murdoch *et al.*, 1986), certain reactive dyes (Docker *et al.*, 1987), plicatic acid (from western red cedar) (Frew *et al.*, 1993), and chloramine T (Bourne *et al.*, 1979).

Not all, and usually only a fraction, of subjects exposed to chemical respiratory allergens will develop occupational asthma. The bases for interindividual differences in susceptibility appear to be governed by both environmental factors and heritable traits. For instance, it has been shown that cigarette smoking is a risk factor for sensitization to at least some chemical respiratory allergens (Venables *et al*., 1989). Genetic associations have also been described (including glutathione *S*-transferase, N-acetyltransferase, and MHC class II polymorphisms), usually with respect to diisocyanate allergy (Mapp *et al*., 2000, 2002; Piirila *et al*., 2001; Wikman *et al*., 2002). What is interesting though is that there appears not to be a consistent association with atopy (that being a predisposition to mount strong immunoglobulin [Ig] E antibody responses) (Venables and Newman Taylor, 1997).

Before considering some of the approaches to hazard identification that have been proposed, it is relevant to highlight two specific areas of uncertainty that have impacted and that continue to impact on research in this area.

The first of these is the route (or routes) of exposure that can result in the development of sensitization of the respiratory tract. It has commonly been assumed that respiratory sensitization to chemicals (in common with sensitization to proteins) is achieved largely, or exclusively, via inhalation exposure to the chemical allergen. However, it needs to be borne in mind that, in common with all other forms of allergic disease, respiratory allergy is dependent upon initiation of an immune response. As adaptive immunity is systemic in nature, there is no reason to assume that skin contact with a chemical respiratory allergen will not result in initiation of the quality of immune response (whatever that is) necessary for effective sensitization of the respiratory tract. There has, for many years, been considerable debate about this issue, but there is perhaps now a growing appreciation that the skin may be a relevant route of exposure for sensitization in humans (Bello *et al*., 2007; Health and Safety Executive, 2000; Isola *et al*., 2008; Kimber, 1996; Kimber and Dearman, 2002b; Redlich, 2010; Redlich and Herrick, 2008; Tarlo and Malo, 2006). Certainly, there is evidence from studies in experimental animals that skin exposure to known chemical respiratory allergens (such as trimellitic anhydride [TMA] and diisocyanates) can cause sensitization of the respiratory tract such that subsequent inhalation challenge with the same chemical will elicit a pulmonary reaction (Botham *et al*., 1989; Karol *et al*., 1981; Rattray *et al*., 1994). This is of more than purely academic interest because the implication is that for effective risk management in the workplace setting and for prevention of respiratory sensitization to chemicals, protection from skin exposure is as important as safeguarding against inhalation.

The other contentious issue is the role of IgE antibody in chemical respiratory allergy (Kimber and Dearman, 2002b; Kimber *et al*., 1998c). It is well established that allergic sensitization to protein antigens (including among others inhalant allergens and food allergens) and the elicitation of immediate-type hypersensitivity reactions are strongly associated with specific IgE antibody. In contrast, it has not been possible to find a consistent relationship between IgE antibody and chemical respiratory allergy. This does not mean that there are no associations, and with some chemical allergens (such as acid anhydrides, reactive dyes, and platinum salts), there do exist correlations between IgE antibody and clinical symptoms (Health and Safety Executive, 1997). A case in point is experience with methyltetrahydrophthalic anhydride (Cullinan, 1998; Kimber *et al*., 1998c; Yokota *et al*., 1998). However, this is not the case with other chemical respiratory allergens and notably with the diisocyanates, where commonly less than half of those with clinically confirmed symptoms have demonstrable IgE antibody (Cartier *et al*., 1989; Cullinan, 1998; Tarlo, 1999; Tee *et al*., 1998; Vandenplas *et al*., 1993). Despite those observations, it has been our contention that the relationship between chemical respiratory allergy/occupational asthma and IgE antibody is actually very much stronger (even with diisocyanates) than is generally acknowledged (Kimber and Dearman, 2002b). There are a number of reasons for this, among the most important being that, for technical reasons, IgE antibody specific for chemical haptens may be missed. Accurate detection depends on the availability of appropriate hapten-protein conjugates for use as substrates, and there is good reason to believe that the effectiveness of antibody binding will vary with the characteristics of the conjugate used and in particular with the molar substitution ratio. It has been argued previously that with very reactive chemicals such as the diisocyanates, there is the ability to react rapidly and extensively with OH, SH, and NH_2 groups on proteins and that this may result in cross-linking, which will compound further the difficulties of accurate measurement (Kimber and Dearman, 2002b). In addition to technical issues, it must be appreciated also that the time of sampling blood for detection of allergen-specific IgE antibodies relative to exposure may impact on antibody detection. It has been shown, for instance, that in serum drawn from patients with confirmed diisocyanate asthma, measurement of IgE antibody was more likely to be successful if samples had been taken within 30 days of the last exposure to the chemical allergen (Tee *et al*., 1998).

The relationship between IgE antibody and chemical respiratory allergy is also of more than simple theoretical interest. There is naturally a wish to base methods for toxicological evaluation and hazard identification on a firm foundation of mechanistic relevance, but certainty about the immunological events and processes that result in the acquisition of respiratory sensitization is lacking. There is no doubt that this has hampered progress and is perhaps one reason why there has historically been reliance on experimental methods (using guinea pigs or rats, but primarily the former) in which the respiratory-sensitizing potential of chemicals is assessed as a function of inhalation challenge–induced pulmonary reactions in previously primed animals. That is, the use of approaches that are based on the elicitation in

animals of reactions that are broadly reflective of occupational asthma in humans and that are independent of an appreciation of relevant immunobiological mechanisms.

A detailed treatise on inhalation challenge models is beyond the scope of this article. Procedural details have varied, but common to most such assays has been an initial immunological priming of animals with the test chemical (using one of several routes of exposure), followed some time later by an inhalation challenge with atmospheres of the same material (Botham *et al.*, 1988, 1989; Karol, 1983; Karol *et al.*, 1981, 1985; Pauluhn, 2003; Pauluhn and Eben, 1991; Pauluhn and Mohr, 1998, 2005; Pauluhn *et al.*, 2002; Sarlo and Clark, 1992; Sarlo and Ritz, 1997). Although such methods offer some benefits, among them being the opportunity to monitor dose-response relationships at the elicitation phase, there are several important disadvantages, including the cost and complexity of atmosphere generation and inhalation challenge.

Given the technical issues and cost implications associated with guinea pig tests, together with the aspiration of developing methods that might confer animal welfare benefits, other strategies for hazard identification have been explored. The approaches that we have been interested in particularly are those that were born of an increased understanding of the nature of immune responses induced by chemical respiratory allergens. The question that fascinated us some years ago was why chemical allergens apparently behave differently. That is, why is it that in humans some chemical allergens preferentially cause skin sensitization and ACD, whereas others are associated selectively with sensitization of the respiratory tract and occupational asthma? Naturally, the distinction is not absolute, and there is evidence that some chemicals (such as, e.g., glutaraldehyde) have the potential in different subjects to induce skin sensitization or sensitization of the respiratory tract. However, there are other chemicals that are much more selective. Thus, for instance, 2,4-dinitrochlorobenzene (DNCB: which we have frequently used as a reference contact allergen) is a very potent skin sensitizer but has never been associated with respiratory allergy. In contrast, the acid anhydrides (including TMA) are associated much more commonly (and sometimes exclusively) with occupational asthma rather than with ACD. These differences are not a function simply of the route of exposure through which chemical is encountered, and our view was that the association of chemical allergens with different forms of allergic disease was likely to be a reflection of the immunological response provoked. So, the intriguing question that we sought to address was why it is that immunogenic small molecular weight chemicals have the potential to cause different types of allergy?

We chose to address this question in mice, and the approach we took was to compare and contrast immune responses induced by contact allergens (including DNCB) and chemical respiratory allergens (including TMA). The experimental approach employed most commonly was to expose mice (usually BALB/c strain) topically to equi-immunogenic concentrations of the test chemicals. In this context, immunogenicity was determined as a function of the vigor of lymphocyte proliferative responses provoked in draining lymph nodes and total lgG antibody production. The strategy was therefore to examine qualitative characteristics of immune responses induced in mice by contact and respiratory chemical allergens under conditions of comparable overall immunogenicity. Although comparative experiments were conducted also following inhalation exposure of mice (Dearman *et al.*, 1991), in most instances, topical exposure was employed. In part, this route of exposure was chosen for technical reasons because administration of chemical allergens to the dorsum of the ears of mice results in immune activation being focused largely in the auricular lymph nodes. The other reason for selection of topical administration was an increasing conviction that in humans encounter with relevant chemical allergens can result in effective sensitization of the respiratory tract.

The basic observation in initial investigations was that under conditions where DNCB and TMA provoked in mice comparable levels of lymph node activation and similar total IgG anti-hapten antibody production, there were qualitative differences in other aspects of the responses observed. Thus, exposure to TMA, but not to DNCB, resulted in an increase in the total serum concentration of IgE. Moreover, the balance between IgG2a and IgG2b anti-hapten antibody isotype production differed. Sensitization to DNCB resulted in preferential induction of IgG2a antibody production, whereas with TMA selectively induced IgG2b responses (Dearman and Kimber, 1991).

These data suggested to us that DNCB and TMA were stimulating in mice the preferential development of discrete functional subpopulations of Th cells. The stimulation by DNCB of IgG2a production and the failure to cause changes in serum IgE were consistent with a preferential Th1 cell phenotype, whereas increases in IgE concentration and preferential IgG2b anti-hapten antibody responses were indicative of a selective Th2 cell phenotype. This interpretation was reinforced and supported further by subsequent investigations with other contact and respiratory chemical allergens and by the measurement of cytokine production by draining LNC prepared from sensitized mice (Betts *et al.*, 2002; Dearman and Kimber, 1991, 1992, 2000; Dearman *et al.*, 1992a, 1994, 1995, 1996a, 2002a, 2005; Kimber and Dearman, 1997a,b, 1998).

The picture that emerged was that with time (over a period of approximately 2 weeks) and following repeated administration of the chemical, topical exposure of mice to contact allergens was associated with development of a preferential Th1-type anti-hapten response characterized by the production of comparatively high levels of IFN-γ and IL-12. Conversely, exposure of mice under the same conditions to chemical respiratory allergens was found to elicit a selective Th2-type response with the production by draining LNC of comparatively high levels of IL-4, IL-5, and IL-13 (classical Th2-type

cytokines) but only low levels of Th1 cytokines (Dearman *et al*., 1995; Kimber and Dearman, 1997a,b, 1998).

This pattern was very much in line with what was emerging about the functional heterogeneity among $CD4^+$ Th cells that had been first described by Mosmann *et al*. (1986). Since that first description of Th cell subsets, there has accumulated a burgeoning literature (Krishnan and Mosmann, 1998; Mosmann and Coffman, 1989; Mosmann and Sad, 1996; Mosmann *et al*., 1991; Romagnani, 2004). The description of Th1 and Th2 $CD4^+$ cells represented an important watershed in the evolution of immunological thinking. For the first time, there was a cellular basis to explain how the adaptive immune system is able to tailor responses to meet the challenges associated with exposure to a particular pathogen. It is clear that the immunological mechanisms that need to be deployed to tackle a viral challenge are very different from those required for resistance to a multicellular parasite, and the Th1/Th2 paradigm provided a model for describing how qualitative differentiation of immune responses might be orchestrated.

Although human Th cell subpopulations do not match exactly those described in mouse, the model has lasted well. However, it has emerged that a Th1/Th2 dichotomy is not the whole story. It became apparent, for instance, that there exists also heterogeneity among $CD8^+$ cytotoxic (Tc) lymphocytes (Kemeny *et al*., 1998; Mosmann and Sad, 1996) and that Tc1/Tc2 cells may contribute to the divergent immune responses induced in mice by contact and respiratory allergens (Dearman *et al*., 1996b, 2005; Moussavi *et al*., 1998). The picture with regard to $CD4^+$ cells has also become complicated further with the description more recently of two other major subpopulations. These are Treg cells as described earlier and Th17 cells. The latter populations are defined by their production of the cytokine IL-17 and are thought to play important roles in allergy (possibly including chemical allergy), autoimmunity, and inflammation (Albanesi *et al*., 1999; Korn *et al*., 2009; Oboki *et al*., 2008; Wang and Liu, 2008). Perhaps even more significantly, there is now evidence that some of these $CD4^+$ cells (Th1, Th2, Th17, and Treg [and possibly others such as Th9]) may not have phenotypes that are as stable as once we assumed. The view that is emerging is that $CD4^+$ T-cell subsets should not be regarded as being terminally differentiated and that in certain circumstances, phenotypic characteristics may change (Locksley, 2009; O'Shea and Paul, 2010; Veldhoen, 2009; Zhou *et al*., 2009; Zhu and Paul, 2010). Although it is likely that such plasticity may provide the immune system with additional flexibility, it is also the case that some of the assumptions made about the role of Th subsets in disease processes, including allergy, may need to be revisited.

The $CD4^+$ and $CD8^+$ T lymphocyte subsets that are believed to contribute to the development and regulation of skin and respiratory sensitization to chemical allergens are summarized in Figure 8.

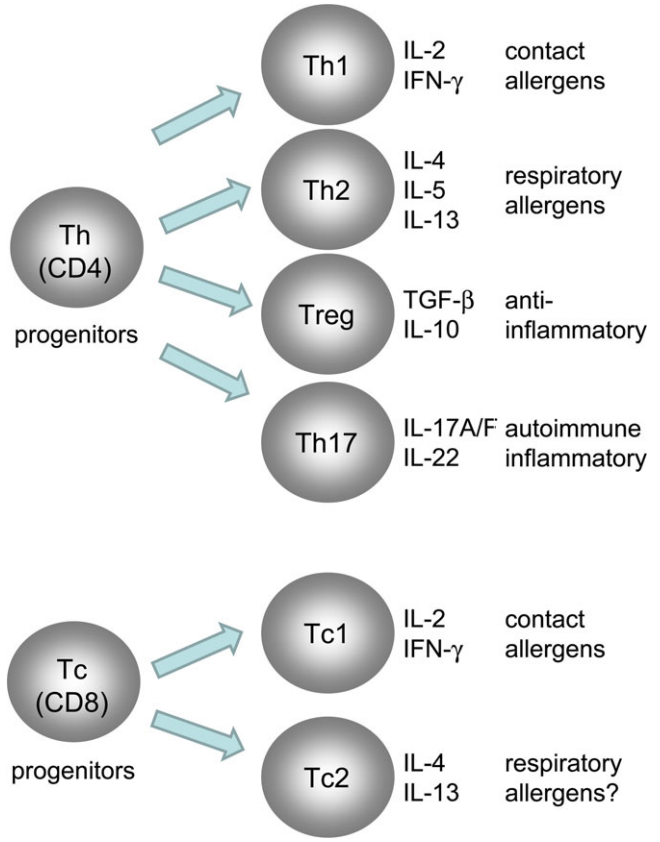

FIG. 8. A diagrammatic summary of the $CD4^+$ and $CD8^+$ T lymphocyte subsets that are believed to contribute to the induction and regulation of skin and respiratory sensitization to chemical allergens.

Despite this complexity, the fact remains that chemical contact and respiratory allergens induce qualitatively distinct immune responses and that this, in turn, translates into different forms of allergic disease. The intriguing question is, therefore, what first triggers these discrete responses. It is possible to speculate at length about that nature of the proximal signals that drive selective Th1- and Th2-type immune responses to chemical allergens, but such speculation is beyond the scope of this article. For brevity, we will highlight here two factors that we believe to be important in shaping allergic responses to chemicals.

The first is the behavior of DC. We have shown that in mice, the kinetics of LC migration induced by topical exposure to chemical allergens differs between contact and respiratory sensitizers (Cumberbatch *et al*., 2005). What we found was that DNCB caused the rapid movement of LC away from the epidermis, whereas the mobilization of LC in response to TMA was significantly slower. The explanation is that TMA, but not DNCB, stimulates the local production within the skin of the anti-inflammatory cytokine IL-10; the argument being that this causes a delay in LC mobilization secondary to an inhibition of TNF-α production (TNF-α being a mandatory signal for LC migration) (Cumberbatch *et al*., 2005). The hypothesis (as yet

unproven) is that the delay in mobilization observed in TMA-sensitized mice and induced by IL-10 causes migratory LC to arrive in draining lymph nodes with a slightly more mature phenotype that favors the stimulation of selective Th2 cell development. The theory is, therefore, that the behavior and mobilization of DC will vary according to the local cytokine and chemokine environment pertaining at the time of encounter with antigen and that this will in turn influence the qualitative nature of immune responses triggered. It has yet to be established whether a delayed tempo of DC migration is a common feature of different families of chemical respiratory allergens.

The other observation of interest is that in some experimental conditions, TMA and DNCB differ with respect to the selectivity of protein binding. Thus, where there are available both cell-associated and soluble proteins, it has been shown that TMA (and other respiratory sensitizers) favor association with soluble proteins, whereas contact allergens preferentially bind to cellular proteins (Hopkins *et al*., 2005). The biological significance of these observations remains uncertain, but the implication is that protein-peptide binding may be an early determinant of the form that allergic responses to chemicals will take.

Despite uncertainty regarding the events that drive qualitative differences, there were two features of murine immune responses to chemical respiratory allergens that appeared to provide a basis for discrimination from contact allergens: the stimulation of an increase in total serum IgE and the elaboration by draining LNC of Th2 cytokines. Both of these were investigated as possible strategies for the prospective identification of respiratory sensitizers.

The first to be considered was induced changes in the serum concentration of IgE. Clearly, an increase in the total serum level of IgE Ig induced by exposure of mice to a chemical respiratory allergen is superimposed upon the elaboration of a specific IgE antibody response (Dearman *et al*., 1992c). As discussed above, the accurate measurement of hapten-specific IgE antibody is technically demanding and dependent upon the availability of an appropriate hapten-protein conjugate. Those technical issues, coupled with the fact that for measurement of hapten-specific antibody levels a separate substrate is required for each chemical considered, make the assessment of specific IgE antibody responses unattractive as an endpoint for hazard identification. However, it is possible to measure with far greater ease and certainty induced increases in total serum IgE levels (using an appropriately calibrated ELISA). This was then the basis for what was called the mouse IgE test.

In this assay, groups of mice (BALB/c strain) were exposed topically on the shaved flanks to the test chemical or to the same volume of the relevant vehicle alone. Seven days later, mice received the same chemical (or vehicle alone) at a reduced concentration on the dorsum of both ears. After a further 7 days, mice were sacrificed and blood drawn for measurement of serum IgE concentrations (Dearman *et al*., 1992b, 2003b; Hilton *et al*., 1995, 1996; Potter and Wederbrand, 1995).

Investigations conducted in one of our laboratories were very encouraging. Chemical respiratory allergens were found to provoke a significant increase in serum IgE, and no such increases were observed with contact allergens (Dearman *et al*., 1992b; Hilton *et al*., 1995). Moreover, these patterns of reactivity were shown to be consistent over time (Hilton *et al*., 1996). In one series of investigations, embracing 15 independent experiments in which TMA and DNCB were compared, it was found that in each instance, TMA caused a significant increase in serum IgE concentrations. In the same experiments, IgE levels in serum drawn from DNCB-treated mice did not differ from vehicle controls (Hilton *et al*., 1996). However, in spite of this early promise, the mouse IgE test was not without difficulties, and this became apparent most clearly during the conduct of an international interlaboratory trial in which variations between mice and between laboratories were recorded (Dearman *et al*., 1998).

Although there remains some enthusiasm for the relative simplicity of the assay, it has to be concluded that it appeared not to "travel" well. From our own experience and from the anecdotal reports of others, one of the issues is that baseline and inducible levels of IgE in mice (and other species) are rather volatile and influenced significantly by a variety of factors. It remains our view that within the confines of a single laboratory environment and where there is opportunity to control housing conditions, diet, and other factors that might impact on the expression of IgE, then the mouse IgE test might provide a robust and reliable approach to hazard identification. However, in the context of developing an assay that could become widely accepted, and that would yield comparable results in independent laboratories, then it was clear that attention should focus elsewhere.

However, before leaving the question of serum IgE as a marker of respiratory-sensitizing potential, it is necessary to consider briefly the use of rats in this regard. For this purpose, the rat strain of choice has been the Brown Norway (BN) that, like the BALB/c mouse strain, has an atopic-like phenotype. The value of the rat, compared with the mouse, is that when using the former, it is possible to take serial bleeds such that kinetic measurements of induced changes in serum IgE concentrations can be made on an individual animal basis. Investigations using BN rats revealed that topical exposure to TMA, but not DNCB, caused an increase in total serum IgE (Arts *et al*., 1997, 1998; Warbrick *et al*., 2002a,b). However, despite the vigor of responses observed with TMA, some other chemical respiratory allergens either failed to provoke an increase in IgE or caused only modest increases (Dearman *et al*., 2003b).

The second feature of immune responses to chemical respiratory allergens that we considered to be of potential utility in the context of hazard identification was the elaboration by draining LNC of Th2 cytokines. Not only did this approach appear attractive from a technical standpoint but it was also relevant from a clinical perspective. Thus, although

there remains a debate about the need for IgE antibody in the development and expression of chemical respiratory allergy and occupational asthma, there is reason to believe that $CD4^+$ Th2 cells play an important, and possibly critical, role (Herrick *et al*., 2003).

The method that is now described as "cytokine profiling" or "cytokine fingerprinting" was based on the observations (summarized earlier) that following topical exposure of mice to chemical contact and respiratory allergens there is, with time, the evolution of preferential Th1-type and Th2-type immune responses, respectively. Such divergent immune responses can be measured conveniently as a function of the differential expression of Th1 and Th2 cytokines.

The conduct of cytokine profiling, procedural details, and aspects of the performance of the assay have been described in detail elsewhere (Dearman and Kimber, 1999, 2001; Dearman *et al*., 1995, 1997, 2002b, 2003a,b, 2007, 2008a, 2009a), and only a brief survey is required here. In the most commonly used configuration of the assay, groups of mice (BALB/c strain) are exposed repeatedly by topical exposure to the test chemical over a period of 13 days. Application concentrations of chemicals are selected on the basis of immunogenicity (activity in the LLNA) when delivered by this route of exposure. Groups of concurrent control mice are treated in identical fashion with TMA or DNCB. One day following the final exposure, animals are sacrificed and lymph nodes excised and pooled for each experimental group. Single-cell suspensions of LNC are prepared under aseptic conditions and cultured for various periods of time. The production by LNC of type 1 cytokines (IL-12 and IFN-γ) and of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) is measured by ELISA (or another appropriate method).

Investigations have been conducted using a wide variety of chemical respiratory allergens (including diisocyanates, acid anhydrides, platinum salts, and glutaraldehyde) and with contact allergens considered not to have the potential to cause respiratory allergy (including, for instance, dinitrofluorobenzene, isoeugenol, and hexyl cinnamic aldehyde). In all instances, the respiratory allergens were associated with selective Th2 cytokine production, whereas in contrast, contact allergens were characterized by preferential type 1 cytokine expression (Dearman and Kimber, 1999, 2001; Dearman *et al*., 1997, 2003a,b, 2007). In our hands, at least, cytokine profiling is a robust and reliable method and remains the assay of choice for the identification of chemical respiratory allergens. Moreover, other investigators have reported similar observations. Although the results of such studies have not always paralleled exactly our own experience, they do serve to confirm that chemical allergens of different classes elicit divergent immune responses at the level of cytokine expression and that exposure of mice to chemical respiratory allergens is normally associated with increased levels of Th2 cytokines (Farraj *et al*., 2006; Goutet *et al*., 2005; Ku *et al*., 2008; Van Och *et al*., 2002; Vandebriel *et al*., 2000, 2003).

Cytokine profiling can be performed also at the level of gene transcription, measuring changes in cytokine mRNA using either reverse transcriptase PCR or the RNAse protection assay (Dearman *et al*., 2008a; Hayashi *et al*., 2001; Manetz *et al*., 2001; Plitnick *et al*., 2002, 2003, 2005; Ryan *et al*., 1998; Selgrade *et al*., 2006; Vandebriel *et al*., 2000, 2003; Warbrick *et al*., 1998). Although this approach has generally been successful with regard to detecting increases in the expression of type 2 cytokines following exposure of mice to chemical respiratory allergens, selective type 1 cytokine responses (and in particular IFN-γ responses) have sometimes proven more difficult to profile. The reasons for the failure in some investigations to detect increases in mRNA for IFN-γ, despite the fact that that this cytokine is clearly actively produced by LNC, are not clear, although it would appear that regulation of this cytokine is effected largely at the level of secretion rather than transcription (Dearman *et al*., 2008a).

Experience to date suggests, therefore, that cytokine profiling provides a reliable approach for identifying potential chemical respiratory allergens and for distinguishing these from contact allergens that lack the potential to cause sensitization of the respiratory tract. It is important to acknowledge that the conduct, interpretation, and overall performance of the assay are dependent upon ensuring that a variety of procedural aspects are adhered to closely and that appropriate concurrent controls are incorporated (Dearman *et al*., 2003a). However, if those experimental conditions are met, then, at present, cytokine fingerprinting probably provides the best option for identification of chemical respiratory allergens.

We have focused here on two approaches to hazard identification (mouse IgE test and cytokine profiling) that have evolved from investigations designed to unravel the mechanisms through which chemicals cause sensitization of the respiratory tract. For the sake of completeness, however, it is important to draw attention to other strategies that are currently being considered. Those other approaches are acknowledged below but are not described in any detail.

In common with skin sensitization, attempts have been made to develop (quantitative) structure activity relationships paradigms for the identification of chemical respiratory allergens. Such approaches are not yet validated and are best regarded currently as being work in progress (Enoch *et al*., 2009, forthcoming; Jarvis *et al*., 1995; Kimber *et al*., 2007; Seed and Agius, 2010; Seed *et al*., 2008). In addition and again in common with skin sensitization, there have been attempts to characterize chemical respiratory sensitizers as a function of changes induced in DC and using other cell- and tissue-based approaches (Corsini *et al*., 2009; Roggen *et al*., 2008). Again, this is work in progress. Finally, as an extension of what has already been achieved with respect to the development of peptide-binding assays for the identification of skin-sensitizing chemicals (Gerberick *et al*., 2004b, 2009), there is interest now in exploring whether a modified approach can be developed for

distinguishing contact allergens from chemical respiratory allergens and for identifying respiratory sensitizers (Lalko *et al*., 2010). This would be predicated on the assumption that skin- and respiratory-sensitizing chemicals display characteristic differences in aspects of their associations with proteins/peptides (amino acid selectivity, kinetics, and/or affinity (Lalko *et al*., 2010; Natsch, 2010).

CONCLUDING COMMENTS

We have come a long way in the last 20 years or more. Progress has been significant, and it is fair to say that with the tools and know-how available now, it is possible to evaluate with some certainty the skin-sensitizing potential of chemicals and to develop accurate assessments of risk. One question presently is how are we placed to address the challenges of developing approaches for characterizing the hazards and risks of skin sensitization without recourse to experimental animals? The answer is that, although progress has been made (as the result of a very substantial investment), there remain many hurdles. Some of these are technical and are likely to be solved soon. Others are less tractable and will require truly innovative approaches. For example, there is a need to define exactly what processes and events determine the potency with which contact allergens cause the acquisition of skin sensitization and how best to model this *in vitro*.

With regard to chemical respiratory allergy, there is still much to be done. A consensus needs to be built regarding the relevant immunobiological mechanisms so that real progress can be made on a broad front in designing new tools and methods.

There is no shortage of intriguing questions in chemical allergy, and these include (in no particular order of importance) (1) the nature of the initial signals that dictate the way in which immune responses to chemical allergens will evolve and the form that allergic disease will take, (2) the bases for interindividual differences in susceptibility to acquisition of skin and respiratory sensitization, and (3) the roles played by various populations of DC in initiating and regulating skin and respiratory sensitization.

The last of those challenges is of particular interest and importance because it can be argued that as the primary cellular orchestrators of adaptive immune responses and residing as they do at the interface between the innate and adaptive immune responses, DC may govern both quantitative and qualitative aspects of sensitization to chemical allergens. One can speculate that a more detailed understanding of the roles played by DC in the recognition, processing, delivery, and presentation of antigen and of their interactions with T lymphocytes may help unlock more of the secrets of chemical allergy. In that context, the substantial complexity of cutaneous DC and the nature of the collective impact of the various DC populations on the induction of immune responses are particularly interesting. One can be certain that such complexity has not arisen by accident and that it is a reflection of a sound biological need. Solving that riddle will not only shed light on the acquisition of chemical allergy but will also make a substantial contribution to our understanding of inflammatory skin disease and pay dividends in the context of vaccine development and other applications.

But that surely is the lasting fascination of toxicology. Not only is it an intriguing discipline in its own right, embracing everything from exploration of the most fundamental biological mechanisms to preservation of human health and environmental safety through the development of accurate risk assessments, but it also offers fascinating and productive alignments with other areas of biomedicine.

It is little wonder therefore that the Society of Toxicology is thriving still after 50 years and continues to go from strength to strength.

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