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Chemical Allergy: Considerations for the Practical Application of Cytokine Profiling

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Chemical respiratory allergy is an important occupational health problem, but there are currently available no validated methods for hazard identification. This is due in part to the fact that the relevant cellular and molecular mechanisms of sensitization of the respiratory tract have been unclear, with particular controversy regarding the role of IgE. There is now increasing evidence that respiratory sensitization is associated with the preferential activation of type 2 T lymphocytes and the expression of type 2 cytokines interleukin (IL)-4, IL-5, IL-10, and IL-13. Type 2 cell products favor immediate type hypersensitivity reactions, serving as growth and differentiation factors for mast cells and eosinophils, the cellular effectors of the clinical manifestations of the allergic responses, and promoting IgE antibody production. There has been considerable interest in the application of cytokine profiling for the characterization of chemical allergens, with cytokine phenotypes analyzed in freshly isolated tissue, or following culture in the presence or absence of mitogen at the level of protein secretion or mRNA expression. Experience to date suggests that the measurement of induced cytokine secretion profiles shows promise for the hazard identification and characterization of chemical respiratory allergens. The purpose of this brief review article is to consider the approaches available and to highlight key procedural issues.

Key Words: **chemical respiratory allergy; hazard identification; IgE; type 2 cell products; cytokine profiling.**

Allergic contact dermatitis, a form of delayed type hypersensitivity (DTH), is one of the most common occupational diseases, and many chemicals have been implicated as causative agents (Cronin, 1980). Exposure to other chemicals, fewer in number, causes allergic hypersensitivity of the respiratory tract, which is also an important occupational health problem (Chan-Yeung and Malo, 1993). For some decades now a number of methods have been available for the prospective identification of chemicals with the potential to cause contact allergy, including those that rely on measurement of challenge-induced elicitation responses, such as the guinea pig maximization test (Magnusson and Kligman, 1970) and the occluded patch test of Buehler (Buehler, 1965). More recently, a method in which contact allergenic potential is assessed as a function of proliferative responses provoked in the induction phase of contact allergy, the murine local lymph node assay (LLNA), has been accepted as a stand-alone method for skin sensitization testing (NIH, 1999). This method is predicated upon an understanding that the initiation of T lymphocyte proliferation in draining lymph nodes is necessary for the acquisition of contact sensitization, and that there exists a close correlation between the vigor of induced proliferative responses and the extent to which skin sensitization develops (Kimber and Dearman, 1991). In contrast, there are currently no well-validated or widely accepted test methods for the prospective identification of chemicals with the potential to cause sensitization of the respiratory tract (Kimber *et al.,* 1996). A major constraint to the development of such tests has been continuing uncertainty regarding the relevant immunobiological mechanisms. Whereas immediate type hypersensitivity responses and asthma to protein allergens are generally acknowledged to be dependent on IgE-mediated mechanisms, there is no such consensus regarding chemical respiratory hypersensitivity (Cullinan, 1998; Mapp *et al.,* 1999). For all known respiratory allergens, specific IgE antibody has been detected in at least some symptomatic patients. For some respiratory sensitizers, including the acid anhydrides, relatively high frequencies (greater than 50%) of specific IgE detection have been reported (Topping *et al.,* 1986). However, particularly in the case of isocyanate-induced asthma, these individuals are often in the minority with as few as 5% of symptom-

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atic patients displaying measurable IgE (Tarlo, 1999; Tee *et al.,* 1998). Despite uncertainty regarding a universal association between IgE antibody and occupational chemical respiratory allergy, there is increasing evidence that allergic sensitization of the respiratory tract will be favored by the induction of a polarized type 2 immune response (Kimber and Dearman, 1997; Mapp *et al.,* 1999). Type 2 cells produce cytokines that facilitate the development and expression of immediate type hypersensitivity reactions, including respiratory sensitization. In common with the development of asthma to protein allergens (Durham *et al.,* 1992; Robinson *et al.,* 1993), eosinophils and T lymphocytes are recruited into the site of inflammation (Bentley *et al.,* 1992) and there is evidence that such lymphocytes selectively express type 2 cytokines (Del Prete *et al.,* 1993; Maestrelli *et al.,* 1994).

With the increased understanding of the immunobiology of chemical respiratory allergy have come new opportunities for the development of tests for the identification of potential respiratory allergens: tests that do not rely upon the elicitation of clinical manifestations of respiratory distress. The aim of this article is to consider the practical application of one such test, cytokine profiling, for the characterization and identification of chemical allergens.

Immunotoxicology of Allergic Responses

Before considering the practical application of cytokine fingerprinting, it is necessary to review briefly the immunological mechanisms that the method attempts to model. Low molecular weight chemical allergens in their native state are unable to induce immune responses and in order to be recognized as foreign by the immune system and to initiate an immune response, a protein-chemical complex must be formed (Landsteiner and Jacobs, 1936). These complexes are internalized and processed for presentation to other cells of the immune system by antigen-presenting cells, with dendritic cells being the most efficient and the only cells that can initiate primary immune responses (Viola *et al.,* 1999). In response to antigen, specific B lymphocytes are stimulated to divide and differentiate into plasma cells that have the machinery to synthesize and secrete specific antibody, including IgE antibody. However, the development of immune responses, including allergic responses, is orchestrated by the activity of $CD4^+$ T helper (Th) cell subpopulations and their cytokine products. In the mature immune response, two phenotypes of Th cell, designated Th1 and Th2, predominate and are distinguished as a function of the cytokines they secrete (Abbas *et al.,* 1996; O'Garra, 1998; Romagnani, 2000). While both populations secrete interleukin (IL) 3 and granulocyte/macrophage-colony stimulating factor (GM-CSF), only Th1 cells express interferon γ (IFN-γ), IL-2, and tumor necrosis factor-β (TNF-β), and only Th2 cells express IL-4, IL-5, IL-6, IL-10, and IL-13. The existence of functional subpopulations of Th cells provides a mechanism for the development of different qualities of immune responses depending upon the nature of the challenge to the host. Th1 cells are adapted for the provision of cell-mediated immunity, whereas Th2 cells produce cytokines that promote humoral immune function and the costimulation and differentiation of B lymphocytes (Abbas *et al.,* 1996; Mosmann and Sad, 1996). This functional dichotomy of Th cells is relevant for the development and expression of allergic disease. Th1 cells are associated with DTH responses, with adoptive transfer of Th1 cell clones sufficient to transfer DTH reactions to naïve recipients (Cher and Mosmann, 1987) and the development of such responses being facilitated by the Th1 cytokine IFN-γ (Diamantstein *et al.,* 1988). Conversely, Th2 cell activation promotes immediate type hypersensitivity reactions, with expression of the type 2 cytokine IL-4 essential for the induction and maintenance of IgE antibody responses (Finkelman *et al.,* 1988b; Kuhn *et al.,* 1991). In contrast, the type 1 cell product IFN-γ inhibits IgE production (Finkelman *et al.,* 1988a). In addition, type 2 cytokines promote other aspects of the immediate type reactions, serving as growth and differentiation factors for mast cells and eosinophils and stimulating the recruitment of eosinophils, cells that play important roles in the elicitation of the clinical manifestations of the allergic response (Kimber and Dearman, 1997; Krishnan and Mosmann, 1998). These polarized forms of Th cell develop with time or following repeated exposure from precursor cells, designated Th0 cells, which have the potential to express both Th1 and Th2 type cytokines, and which themselves develop from primitive precursor (Thp) cells that express IL-2 only (Bendelac and Schwartz, 1991; Mosmann *et al.,* 1991). The differentiation of Th1 and Th2 cells is controlled largely by positive feedback and negative cross-regulation by Th1 and Th2 cell products (Krishnan and Mosmann, 1998). The cytokines that appear to be of greatest significance in polarizing the immune response are the type 2 cell product IL-10, and IL-12, a heterodimeric cytokine produced by dendritic cells and some epithelial cells. The source of the cytokines that drive the initial development of divergent Th cell phenotypes is still a matter for some debate, but it has been suggested that the cellular components of the innate immune system, including mast cells, may play a role (Kimber and Dearman, 1997). Therefore it is the balance between Th1 and Th2 cytokines that determines the direction and nature of the induced immune response.

Although attention has focused on the role of Th cells and their products in the development of allergic responses, it has become apparent that $CD8^+$ T cytotoxic (Tc) cells may also play influential roles. Two populations, designated Tc1 and Tc2, have been described that display selective cytokine secretion patterns analogous with Th1 and Th2 cells, respectively (Mosmann and Sad, 1996). Although there is limited evidence of a physiological role for Tc2-type cells (Kimber and Dearman, 1997), there is increasing interest in the possibility that Tc1-type cells that produce IFN-γ may mediate and/or regulate contact hypersensitivity reactions (Kimber and Dearman, 2002). Until recently, allergic contact dermatitis, in common

with other forms of DTH reaction, was viewed as a Th1-type response, mediated by $CD4^+$ IFN-γ producing effector cells. However, there is a growing appreciation that, in contrast to DTH reactions to complex protein or cellular antigens, $CD8^+$ effector cells may play important roles in the development and elicitation of allergic contact dermatitis to chemical allergens (Gorbachev and Fairchild, 2001). Allergic contact dermatitis to chemicals is apparently associated with the preferential activation of Th1 and Tc1 cells, whereas immediate type hypersensitivity reactions such as chemical respiratory hypersensitivity are favored by the selective activation of Th2 (and possibly Tc2) type cells.

Cytokine Profiling of Chemical Allergens: Background to Method Development

The observation that topical exposure of BALB/c strain mice to chemical contact and respiratory allergens stimulates different qualities of immune response with respect to IgE and IgG antibody responses (Dearman and Kimber, 1991) led us to question whether it might be possible to characterize allergens as a function of cytokine secretion profiles. Under conditions of exposure of equivalent immunogenicity with respect to lymphocyte proliferation and IgG antibody responses, the cytokine phenotypes induced by the contact allergen 2,4-dinitrochlorobenzene (DNCB) and trimellitic anhydride (TMA) were examined (Dearman *et al.,* 1997). Draining lymph node cells (LNC) excised from DNCB-treated mice expressed high levels of the type 1 cytokine IFN-γ, but relatively low levels of the type 2 cytokines IL-4 and IL-10. The converse type 2 cytokine secretion profile was provoked by topical exposure to TMA (Dearman *et al.,* 1994). Interestingly, these polarized cytokine phenotypes take time to mature, with LNC isolated three days after initiation of exposure displaying a mixed Th0-like phenotype with type 1 and type 2 cytokines expressed following treatment with both chemical contact and respiratory allergens (Betts *et al.,* 2002; Dearman *et al.,* 1994; Moussavi *et al.,* 1998). A more prolonged (13-day) exposure protocol was required for the development of a differentiated cytokine phenotype (Betts *et al.,* 2002; Dearman *et al.,* 1994, 1997). The relative contribution of $CD4^+$ and $CD8^+$ cells to the cytokine phenotype of allergen-activated cells has been examined using negative (complement depletion) and positive selection (antibody coated magnetic beads) techniques (Dearman *et al.,* 1996; Moussavi *et al.,* 1998). These experiments demonstrated that early (three days) following initiation of exposure to either DNCB or TMA, the mixed cytokine phenotype observed was a function of IFN-γ-secreting $CD4^+$ (Th1) and $CD8^+$ (Tc1) cells and IL-4-secreting $CD4^+$ (Th2) cells (Moussavi *et al.,* 1998). The preferential type 2 cytokine secretion profile observed after prolonged treatment with the respiratory sensitizer TMA was due to the activation of $CD4^+$ (Th2) cells, with the relatively low levels of IFN-γ derived exclusively from $CD8^+$ (Tc1) cells. The selective type 1 cytokine expression pattern stimulated by more chronic exposure to DNCB was associated with both $CD4^+$ (Th1) and $CD8^+$ (Tc1) IFN-γ-expressing cells, with the low levels of type 2 cytokines being a result of $CD4^+$ (Th2) cell activation (Dearman *et al.,* 1996). These data suggest that as the immune response to a chemical allergen matures with time and becomes polarized, exposure to the contact allergen DNCB is associated with the selective development of Th1 and Tc1 cells, whereas treatment with the respiratory allergen TMA is associated primarily with Th2 cell development.

Initial Evaluation of Cytokine Profiling

Subsequent investigations using a range of additional chemical respiratory allergens, including isocyanates, platinum salts, glutaraldehyde, and various acid anhydrides, have confirmed that the expression of a preferential type 2 cytokine expression profile is a general property of respiratory sensitizing chemicals (Dearman and Kimber, 2001). Using the same exposure protocol as that used for the chemical respiratory allergens described above, treatment with allergens such as 2,4-dinitrofluorobenzene (DNFB), isoeugenol, and hexyl cinnamic aldehyde (contact allergens that apparently lack respiratory sensitizing activity) stimulates instead a selective type 1 cytokine secretion pattern (Dearman and Kimber, 2001). For selected respiratory allergens, including the acid anhydrides phthalic anhydride (PA) and maleic anhydride, and the isocyanates diphenyl methane diisocyanate (MDI) and fluorescein isocyanate, it has been confirmed that the induced preferential type 2 cytokine expression profile is a function of Th2 ($CD4^+$) cell activation exclusively (Dearman and Kimber, 2000; Dearman *et al.,* 1996, 2002a).

Cytokine Profiling in Practice: Considerations of Dose Selection and Controls

These data demonstrate that for a variety of chemical allergens, the measurement of induced secretion of IFN-γ and IL-12 provide preferential markers of type 1 responses, whereas IL-4, IL-5, IL-10, and IL-13 can be used as markers of selective type 2 responses. In the experiments described above, LNC have been pooled on an experimental group basis and single cell suspensions prepared for culture. This is in order to provide for a sufficient yield of LNC for the analysis of the complete range of cytokines that would not be possible if LNC were prepared on an individual animal basis. For the reference contact allergen DNCB and respiratory allergen TMA, despite some interexperimental variations in absolute levels of cytokine, analysis of cytokine phenotypes revealed that the ability of DNCB and TMA to induce Th1 and Th2 cytokine profiles, respectively, was very consistent. Statistical analyses (Student's *t*-test) of four independent experiments demonstrated that differences in cytokine expression patterns between DNCB and TMA-activated LNC were significant at the 5% (IFN-γ, IL-12, and IL-10) or 1% (IL-4, IL-5, and IL-13) levels

(Dearman and Kimber, 2001). Since the phenotypes of DNCB- and TMA-stimulated LNC were invariably Th1- and Th2-like, respectively, it is our practice to examine the cytokine secretion profiles induced by test chemicals concurrently with DNCB and TMA. These chemicals provide negative and positive controls for respiratory sensitizing potential and with respect to respiratory sensitization hazard characterization, a chemical is considered to have significant potential for respiratory allergenicity if similar levels of type 2 cytokines are induced to those observed for the concurrent TMA control.

Another issue regarding the application of this method in practice is that of dose selection for test chemicals. It is necessary that concentrations are used that are known to be immunogenic and to stimulate a cutaneous immune response of the vigor necessary for measurement of cytokine secretion. Currently, dose selection is based in prior conduct of an LLNA, a predictive test for the identification of skin sensitizing chemicals in which activity is measured as a function of LNC proliferative responses induced by topical exposure of mice to the test material (Kimber and Dearman, 1991; NIH, 1999). In addition to assisting with dose setting for subsequent cytokine fingerprinting, the purpose of conducting a prior LLNA is to evaluate the overall allergenic properties of the test chemical. The rationale for this is that in our experience those chemicals that are able to cause allergic sensitization of the respiratory tract also elicit positive responses in guinea pig and mouse predictive tests for skin sensitization (including the LLNA; Kimber, 1995). This is something of a paradox because many of those same chemical respiratory allergens do not, or only rarely, cause allergic contact dermatitis in humans. The reason for this is unclear, but in practice the fact remains that chemical respiratory allergens (and contact allergens) will be identified in tests for skin sensitization such as the LLNA. The corollary is that chemicals that fail to elicit positive responses in the LLNA are very unlikely to have a significant potential to cause respiratory sensitization. On this basis it seems reasonable to perform a preliminary LLNA and to determine cytokine fingerprinting patterns only with those chemicals that yield positive responses. It is then possible to select the appropriate doses for cytokine fingerprinting based on an understanding of application concentrations that provoked positive LLNA responses.

Independent Applications of Cytokine Profiling

The observation that different classes of chemical allergen stimulate divergent immune responses at the level of cytokine expression has been confirmed by other investigators (Hayashi *et al.,* 2001; Manetz *et al.,* 2001; Vandebriel *et al.,* 2000; van Och *et al.,* 2002). In apparent conflict with these data are reports that contact allergens such as DNCB and oxazolone can stimulate type 2 cytokine expression (Ulrich *et al.,* 2001). Leaving aside for the moment the fact that in these latter experiments cytokine production was measured following the *in vitro* restimulation of allergen-activated LNC with the polyclonal T cell mitogen anti-CD3 antibody, the important point is that the divergent cytokine secretion profiles provoked by different classes of chemical allergen are selective, not absolute. As discussed previously, it is the balance between Th1 and Th2 cell activation and cytokine products that determines the nature of the developing immune response. Thus, the observation by Ulrich and colleagues (2001) that contact allergens such as DNCB stimulated measurable IL-4 expression is not inconsistent with the results reported herein, as spontaneous expression of type 2 cytokines was also detected after treatment with DNCB, albeit at much lower levels than those stimulated by TMA (Dearman *et al.,* 1997; Hayashi *et al.,* 2001). However, it is not possible in the experiments conducted by Ulrich *et al.* (2001) to compare directly the ability of DNCB and TMA to provoke selective type 1 and type 2 cytokine secretion patterns since the concentration of DNCB used was considerably less immunogenic than was the dose of TMA selected.

Considerations of Strain and Species

In the majority of the experiments described above, BALB/c mice have been used (Dearman and Kimber, 2001; Hayashi *et al.,* 2001; Manetz *et al.,* 2001; Ulrich *et al.,* 2001; Vandebriel *et al.,* 2000; van Och *et al.,* 2002). Mice of this strain are known to be high IgE responders and are predisposed to make Th2-type responses. It could be argued that the use of the BALB/c strain mouse may have biased the observed cytokine phenotype towards type 2 responses. It must be remembered that concurrent exposure of this mouse strain to contact allergens such as DNCB under conditions of equivalent immunogenicity provokes selective type 1 cytokine responses (Dearman and Kimber, 2001; Hayashi *et al.,* 2001; Manetz *et al.,* 2001; Vandebriel *et al.,* 2000; van Och *et al.,* 2002). Furthermore, recent investigations have demonstrated that in a mouse strain that is more predisposed to make type 1 responses, similar divergent patterns of cytokine expression are elicited by DNCB and TMA (Hayashi *et al.,* 2001). C57BL6 strain mice are reported to be biased towards type 1 responses (Guler *et al.,* 1996), however, exposure of this strain of mice to respiratory allergens (including TMA, PA, and MDI) also stimulated a preferential type 2 cytokine response, whereas contact allergens such as DNCB and oxazolone induced a selective type 1 cytokine phenotype (Hayashi *et al.,* 2001). In some experiments, an alternative rodent strain has been employed, the Brown Norway (BN) rat, which in common with the BALB/c strain mouse is regarded as having a predisposition to type 2 responses. Topical exposure of BN rats to DNCB or to TMA under conditions of equivalent immunogenicity with respect to induced proliferative responses resulted in significant increases in total serum IgE concentration being recorded only following treatment with TMA (Arts *et al.,* 1997). In addition, measurement of cytokine secretion profiles provoked in draining LNC

by topical exposure of BN rats to DNCB or to TMA revealed the selective induction of type 1 and type 2 cytokine patterns, respectively (Dearman *et al.,* 2002b). Taken together, these data suggest that chemical contact allergens such as DNCB and respiratory sensitizers such as TMA exhibit an innate ability to stimulate type 1 and type 2 cytokine production profiles, respectively, which are species and strain independent.

Route of Exposure: Is the Skin Appropriate?

Another important issue is that of route of exposure. In the experiments described above, rodents have been treated with chemical allergen epicutaneously and there are reports in the literature that prolonged topical exposure and repeated applications of chemical allergen may result in a bias towards Th2-type responses (Kitagaki *et al.,* 1999). However, the exposure regimen utilized in the majority of the experiments described above has been designed to induce polarized cytokine secretion profiles following topical exposure to different classes of chemical allergen (Dearman and Kimber, 2001; Hayashi *et al.,* 2001; Manetz *et al.,* 2001). Thus, repeated topical application over a 13-day period of the known human contact allergen DNCB results in the development of a selective type 1 cytokine phenotype (Dearman and Kimber, 2001; Hayashi *et al.,* 2001; Manetz *et al.,* 2001). By definition, the skin represents the relevant route of exposure for contact sensitization; there is reason to believe, however, that dermal exposure may be relevant also for induction of effective sensitization of the respiratory tract. Although inhalation is undoubtedly the most important and most common route of exposure to chemical respiratory allergens, there is experimental evidence and some limited clinical evidence to suggest that sensitization of the respiratory tract may be induced following skin contact (Kimber and Dearman, 2002). Further it has been demonstrated that similar qualities of immune response with respect to antibody isotype profiles are stimulated in BALB/c strain mice regardless of whether sensitization is via inhalation or the skin, with only the respiratory allergen TMA inducing IgE antibody expression (Dearman and Kimber, 1991; Dearman *et al.,* 1991). Taken together with the fact that the dermal route of exposure facilitates analyses of cytokine responses induced in discrete draining lymph nodes, our accumulated experience confirms that the characterization of cytokine responses induced following topical application of different classes of chemical allergen is an appropriate strategy for hazard identification.

Requirement for Restimulation *in Vitro*?

One of the protocol variations that has been explored by several investigators is to measure cytokine expression (either at the protein secretion or mRNA level) following restimulation of LNC with mitogen *in vitro* (Manetz *et al.,* 2001; Ulrich *et al.,* 2001; Vandebriel *et al.,* 2000; van Och *et al.,* 2002). Although it is necessary to restimulate LNC with the T cell mitogen concanavalin A (con A) in order to detect expression of IL-4 protein, there is sufficient spontaneous production of the other cytokines (IFN-γ, IL-5, IL-10, IL-12, and IL-13) for measurement by enzyme-linked immunosorbent assay (ELISA) in the absence of restimulation (Dearman *et al.,* 1994, 2002a,b). Mitogen activation per se does not stimulate the expression of IL-4. Thus, cells derived from naive or vehicle-treated mice fail to secrete measurable levels of IL-4, despite the presence of mitogen (Dearman *et al.,* 2002b). Other authors have described similar difficulties in the detection of IL-4 and have suggested that such may be due to the uptake of this cytokine in autocrine fashion, given that measurement of IL-4 is more efficient when reutilization of this cytokine is blocked by antireceptor antibodies (Bancroft *et al.,* 1998). There is some evidence to suggest that the addition of mitogens such as polyclonal anti-CD3 antibody may bias the cytokine secretion profile with respect to cytokines other than IL-4; certainly the experience of Ulrich *et al.* (2001) that contact allergens induce type 1 and type 2 cytokine expression may be explained partly by the incorporation of a mitogen restimulation step. Indeed, other investigators have shown that stimulation of naïve spleen cells with anti-CD3 antibody is sufficient to induce detectable cytokine production (Wang *et al.,* 1992). In contrast, with the exception of IL-12 protein, which is produced constitutively, quiescent control LNC do not generally produce detectable levels of other cytokines (IFN-γ, IL-4, IL-5, IL-10, and IL-13) in the absence of mitogen restimulation, suggesting that the measurement of cytokine expression in the absence of mitogen may be more selective for allergen-induced effects (Betts *et al.,* 2002; Dearman *et al.,* 1994, 2002a). Under some circumstances restimulation with mitogen may not compromise completely the development of polarized cytokine phenotypes. Culture of LNC isolated five to seven days after initiation of exposure to different classes of chemical allergen with the T cell mitogen con A does result in the induction of divergent cytokine expression profiles (Vandebriel *et al.,* 2000; van Och *et al.,* 2002). However, such cytokine secretion patterns are less polarized than those derived following measurement of spontaneous cytokine production, particularly with respect to IFN-γ expression, suggesting that analysis of cytokine profiles in the absence of restimulation may provide a more robust assessment of sensitizing hazard.

Message or Protein?

One other area of significant variation among cytokine profiling protocols is whether cytokine responses are measured as a function of protein secretion, or at the level of mRNA expression. Cytokine mRNA expression has been examined by reverse transcription polymerase chain reaction (RT-PCR; Hayashi *et al.,* 2001; Ryan *et al.,* 1998; Vandebriel *et al.,* 2000; Warbrick *et al.,* 1998) or by ribonuclease protection assay (RPA; Betts *et al.,* 2002; Manetz *et al.,* 2001; Plitnik *et al.,* 2002). Measurement of cytokine transcripts by RT-PCR re-

vealed that in the absence of mitogen restimulation *in vitro*, prolonged exposure to TMA induced increased levels of IL-4 mRNA expression compared with treatment with DNCB (Hayashi *et al.,* 2001; Ryan *et al.,* 1998; Warbrick *et al.,* 1998). The reverse pattern of cytokine mRNA expression was recorded for the type 1 cytokines IFN-γ (Warbrick *et al.,* 1998) and IL-12 (Hayashi *et al.,* 2001), although it must be noted that in the latter studies and in other studies, IFN-γ mRNA levels did not discriminate between contact and respiratory allergens (Hayashi *et al.,* 2001; Vandebriel *et al.,* 2000). Several studies have now been published in which allergen-induced changes in cytokine gene expression have been analyzed by RPA, a technique with a somewhat lower level of sensitivity than RT-PCR (Betts *et al.,* 2002; Manetz *et al.,* 2001; Plitnik *et al.,* 2002). This method is sufficiently sensitive to detect consistently preferential type 2 cytokine expression following prolonged treatment with the respiratory allergens TMA and toluene diisocyanate (TDI), with particularly high levels of transcripts for IL-4 and IL-10 detected in freshly isolated tissue (Betts *et al.,* 2002; Manetz *et al.,* 2001; Plitnik *et al.,* 2002). Without restimulation *in vitro* with T cell mitogen con A, RNA isolated from DNCB- or DNFB-activated LNC tissue did not express a selective type 1 cytokine mRNA phenotype, particularly with respect to IFN-γ (Manetz *et al.,* 2001; Plitnik *et al.,* 2002). The reasons for the failure to detect increased mRNA for IFN-γ despite robust secretion of this cytokine are presently unclear, although it would appear that production of this cytokine by draining LNC is controlled mainly at the level of secretion. The measurement of cytokine mRNA levels in freshly isolated tissue by RPA offers a number of potential advantages compared with other methods for cytokine analysis, including the lack of requirement for tissue culture or restimulation, the simultaneous measurement of multiple cytokines and the possibility of evaluating cytokine responses at the individual animal level, given the relatively small amounts of tissue used. It should be noted that to date analysis of cytokine expression by RPA has only been performed on tissue derived following treatment with a very limited number of chemical allergens (Betts *et al.,* 2002; Manetz *et al.,* 2001; Plitnik *et al.,* 2002). These experiments revealed that whereas the two respiratory allergens tested could be identified on the basis of increased levels of mRNA for type 2 cytokines in freshly isolated tissue, there was no measurable induction by contact allergens of transcripts for type 1 cytokines in the absence of restimulation (Betts *et al.,* 2002; Manetz *et al.,* 2001; Plitnik *et al.,* 2002).

With respect to the characterization of sensitizing hazard, the judicious view is that cytokine profiling by RPA and by RT-PCR may identify those chemicals with respiratory sensitizing potential as a function of induced type 2 cytokine expression. More experience is required with a wider range of respiratory sensitizers before the sensitivity and selectivity of this method versus measurement of protein secretion by ELISA can be assessed. However, as both methods are less effective at measuring the expression of type 1 cytokines such as IFN-γ than is measurement of protein secretion by ELISA, they have less utility for the identification of contact allergens and the discrimination between respiratory and contact allergens.

Interexperimental Variation

Another important issue is that of interexperimental variation. In the most commonly applied configuration of cytokine profiling (Betts *et al.,* 2002; Dearman and Kimber, 2001; Dearman *et al.,* 2002a), as described previously, LNC are pooled on an experimental group basis and cultured for preparation of supernatants and analysis of cytokine protein secretion by ELISA. It is not possible therefore to examine interanimal variations in cytokine production patterns. However, as described in a previous section (Cytokine Profiling in Practice: Considerations of Dose Selection and Controls), the statistical significance of interexperimental differences in cytokine secretion phenotypes of DNCB- and TMA-activated LNC has been addressed. These studies demonstrated that DNCB-stimulated LNC expressed significantly higher levels of the Th1-type cytokines IFN-γ and IL-12 under conditions where TMA-activated LNC produced significantly higher levels of the Th2-type cytokines IL-4, IL-5, IL-10, and IL-13 (Dearman and Kimber, 2001; Dearman *et al.,* 2002b). Using an alternative method in which LNC are restimulated with mitogen prior to analysis of cytokine protein production by ELISA, lymph nodes were pooled on an individual animal basis following exposure to allergen, allowing assessment of interanimal variation in cytokine expression, although insufficient cells were available from vehicle-treated lymph nodes to perform these analyses (Vandebriel *et al.,* 2000). However, the addition of mitogen, as described previously, results in cytokine secretion profiles for DNCB- and TMA-activated cells that are less polarized than those observed following culture of LNC in the absence of mitogen (Dearman and Kimber, 2001; Vandebriel *et al.,* 2000). The addition of mitogen, notwithstanding the advantage of provision for analysis of cytokine expression at an individual animal level, results in a considerable loss of sensitivity.

Recent advances in methodology for the measurement of cytokine proteins have made it possible to analyze the expression of multiple cytokines in small sample volumes, facilitating the measurement of cytokine secretion patterns at the individual animal level in the absence of restimulation. Systems such as the Bio-Plex™ cytokine assay (Bio-Rad, Hercules, CA) are based on a similar principle to a capture sandwich ELISA, but anticytokine antibodies are covalently coupled to dyed polystyrene beads in a microtiter format. Relatively small volumes of sample (50 μl) are required and bound cytokine is visualized using biotinylated detection antibody and phycoerythrin-labeled streptavidin. By reading each individual bead in the mixture, the analytical system can discriminate between up to 100 different proteins including cytokines. Figure 1 displays the cytokine secretion profiles for LNC derived from DNCB-

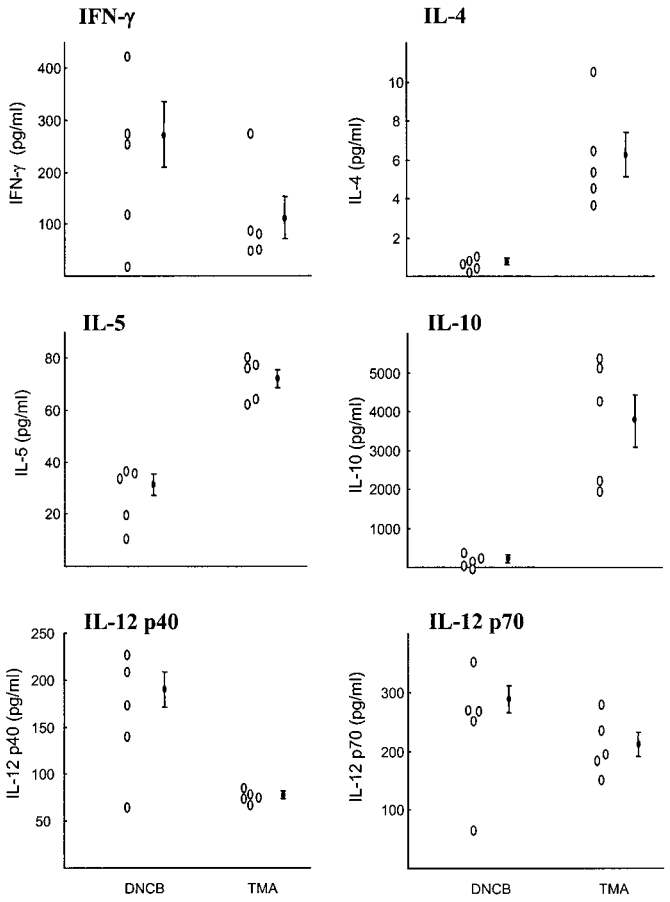

FIG. 1. Cytokine profiling of chemical allergens: interanimal variation. Draining auricular lymph node cells (LNC) were isolated 13 days after the initiation of topical exposure of BALB/c strain mice ($n = 5$) to 1% 2,4-dinitrochlorobenzene (DNCB) or to 10% trimellitic anhydride (TMA), each dissolved in acetone:olive oil (4:1) vehicle. Lymph nodes were pooled on an individual animal basis, a single cell suspension was prepared by mechanical disaggregation and cells were cultured at 10^7 cells/ml for 120 h. Concentrations of interleukin (IL) 4, IL-5, IL-10, IL-12 p40, IL-12 p70, and interferon γ (IFN-γ) were measured in supernatants using the Bio-Plex™ cytokine array system (Bio-Rad, Hercules, CA). Individual animal data are shown, together with mean and SEM of cytokine expression. The statistical significance of differences in cytokine expression by DNCB- and TMA-activated LNC was evaluated by Student's *t*-test (IFN-γ, $*p < 0.05$; IL-4, IL-5, IL-10, and IL-12 p40, $**p < 0.01$; IL-12 p70, not significantly different).

and TMA-exposed mice ($n = 5$), where lymph nodes have been pooled on an individual animal basis and cultured for 120 h in the absence of mitogen. Production of the type 1 cytokines IFN-γ and IL-12 (p40 and p70 subunits) and the type 2 cytokines IL-4, IL-5, and IL-10 have been analyzed using the Bio-Plex cytokine array system. These experiments revealed that there was remarkably little interanimal variation in cytokine expression, with very marked type 2 cytokine secretion profiles observed for LNC isolated from each of the TMA-exposed mice, and the converse type 1 cytokine pattern recorded for LNC derived from each of the DNCB-treated animals. In this particular experiment shown, one of the DNCB-treated mice exhibited somewhat lower levels of both IL-12 (p40 and p70) and IFN-γ expression than did the other DNCB-treated animals. Even so, between group comparisons revealed that LNC derived from DNCB-exposed animals produced significantly more IFN-γ ($p < 0.05$) and IL-12 p40 ($p < 0.01$) than did those isolated from TMA-treated mice, whereas exposure to TMA stimulated significantly higher levels of IL-4, IL-5, and IL-10 ($p < 0.01$). Interestingly, this system was also sensitive enough to detect IL-4 secretion in the absence of restimulation with mitogen. These data suggest that there is little interanimal variation in the ability of DNCB and TMA to elicit distinct type 1 and type 2 cytokine secretion profiles and demonstrate that similar cytokine production patterns are detected regardless of whether lymph nodes are pooled on a group basis or individual animals are assessed.

Concluding Comments

Substantial progress has been made with respect to the understanding of the immunotoxicology of chemical respiratory allergy. There is an increasing consensus that this important occupational disease is a result of the induction and activation of preferential type 2 T lymphocyte responses following exposure to chemicals with respiratory sensitizing potential. There has been interest in the application of cytokine profiling to the identification and characterization of chemical allergy, with cytokine phenotypes analyzed in freshly isolated tissue or after culture in the presence or absence of mitogen at the level of protein secretion or mRNA expression. The most common configuration of cytokine fingerprinting (measurement of induced cytokine secretion patterns in the absence of restimulation) in particular shows considerable promise as an approach for the identification of chemical respiratory allergens.

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