

Allergen-induced cytokine phenotypes in mice: role of CD4⁺ and CD8⁺ T cell populations

R. J. Dearman, N. Humphreys, R. A. Skinner and I. Kimber

Syngenta Central Toxicology Laboratory, Macclesfield, Cheshire, UK

Summary

Background CD4⁺ T cells expressing type 2 cytokines have been implicated in the pathogenesis of asthma to high-molecular-weight allergens. Topical exposure of BALB/c strain mice to low-molecular-weight chemical contact and respiratory allergens stimulates type 1 and type 2 cytokine secretion phenotypes, respectively.

Objective To examine the relative frequencies of cytokine-positive CD4⁺ and CD8⁺ T cells and their contributions to these cytokine secretion profiles.

Methods Draining auricular lymph nodes were isolated 13 days after initiation of topical exposure of female BALB/c strain mice to chemical allergen, or to vehicle alone. The frequency of intracellular cytokine (IL-4 and IFN- γ)-positive CD4⁺ and CD8⁺ lymphocytes was enumerated by flow cytometry. The relative contribution of CD4⁺ and CD8⁺ cells to cytokine secretion profiles was assessed by negative selection.

Results Exposure to allergen resulted in an increased frequency of both IFN- γ ⁺ CD4⁺ and CD8⁺ lymphocytes, although there were no marked differences between trimellitic anhydride (TMA)- and 2,4-dinitrochlorobenzene (DNCB)-activated lymph node cells. Treatment with TMA induced approximately five times as many IL-4⁺ CD4⁺ cells as did exposure to DNCB. This pattern of cytokine staining was also observed for a further pair of contact and respiratory allergens; respectively, formalin and fluorescein isothiocyanate.

Conclusion These data demonstrate that the divergent immune responses induced in mice by different classes of chemical allergen are independent of changes in the frequency of IFN- γ ⁺ cells, but are associated with differential frequencies of IL-4-expressing CD4⁺ T cells.

Keywords cytokines, flow cytometry, mouse model, T cell subsets, respiratory allergy

Submitted 22 June 2004; revised 1 November 2004; accepted 17 January 2005

Introduction

Although it is generally accepted that IgE antibody is the principal biological effector of immediate-type hypersensitivity responses induced by protein allergens, there is no similar consensus regarding sensitization of the respiratory tract to low-molecular-weight chemical allergens such as acid anhydrides (including trimellitic anhydride (TMA)), isocyanates, certain reactive dyes and some platinum salts [1–3]. For some chemical respiratory sensitizers (e.g. acid anhydrides), greater than 50% of symptomatic patients may display measurable specific IgE [4, 5]. For other allergens, particularly for some isocyanates, lower frequencies (5–20%) have been reported [6, 7]. There is increasing evidence, however, that chemical respiratory sensitization will also be favoured by a polarized type 2 immune response [8, 9], with reports that eosinophils

and T lymphocytes with a selective type 2 cytokine phenotype are recruited into the site of inflammation [10, 11].

The initial observations of cytokine polarized T lymphocyte subpopulations with type 1 or type 2 cytokine profiles were confined to the CD4⁺ subset [12]. It is now apparent that CD8⁺ T cells also display functional heterogeneity and differentiated cytokine expression. The populations designated type 1 express preferentially IL-2 and IFN- γ , whereas type 2 subsets secrete IL-4, IL-5, IL-10 and IL-13 [13]. There is considerable evidence to support a role for the involvement of CD4⁺ type 2 cells both in atopic allergic asthma and in non-atopic and occupational asthma [9, 14]. For example, in tissue samples from asthma patients, bronchial mucosal expression of IL-4 and IL-5 mRNA co-localized to CD3⁺ T cells, the majority of which were CD4⁺ [15]. Furthermore, allergen-induced IL-5 secretion by peripheral lymphocytes isolated from atopic patients with asthma or rhinitis compared with non-atopic control subjects was found to be dependent upon CD4⁺, but not CD8⁺, T cells [16]. CD8⁺ T cells may influence also allergic respiratory hypersensitivity reactions. In several studies IFN- γ - or IFN- γ -secreting CD8⁺

Correspondence: Rebecca J. Dearman, Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, UK.

E-mail: rebecca.dearman@syngenta.com

cells, but not type 2 CD8⁺ cells, have been identified in the peripheral blood of allergic patients [17, 18]. There are other reports suggesting that equivalent frequencies of peripheral blood CD4⁺ and CD8⁺ T cells producing type 1 and type 2 cytokines are observed in asthmatic subjects [19].

There is little information available as to the likely contributions of these cell types to the development of chemical respiratory sensitization in humans although it has been reported that all T cell clones generated from the peripheral blood of isocyanate-sensitized workers were IFN- γ -secreting CD8⁺ cells, some of which also expressed IL-5 [20]. Rodent models have been established to characterize the immune response to chemical respiratory sensitizers and may be useful for the prospective identification of the respiratory sensitizing hazard of new chemicals. In such experiments, the acid anhydride TMA has been utilized as a reference chemical respiratory allergen as it is a relatively common cause of occupational immunological lung disease in which patients may present with early and/or late onset asthma and rhinitis or with late respiratory systemic syndrome, a disease similar to hypersensitivity pneumonitis [21]. Thus, topical exposure of BALB/c strain mice to TMA, results in the induction of a selective type 2 cytokine secretion profile [22, 23]. It was demonstrated by negative selection that the type 2 cytokines were associated exclusively with CD4⁺ T cells, whereas low level IFN- γ expression was a property of CD8⁺ T cells [23, 24]. Identical exposure to skin sensitizing chemicals such as 2,4-dinitrochlorobenzene (DNCB), that cause delayed-type hypersensitivity reactions but lack the potential to cause respiratory sensitization, elicits the converse type 1 cytokine secretion profile, with high levels of IFN- γ derived from both CD4⁺ and CD8⁺ T cells [22–24]. In these experiments, the topical route of exposure rather than exposure by inhalation was selected because of the accessibility and extent of activation of the lymph nodes that drain the site of application.

The evidence to date suggests that both CD4⁺ and CD8⁺ cell subsets may play influential roles in chemical respiratory allergy. In the current series of experiments, cytokine-positive CD4⁺ and CD8⁺ cells induced by different classes of chemical allergen have been enumerated using intracellular cytokine staining. The identity of cytokine-secreting cells has been confirmed by negative selection.

Materials and methods

Animals

Female (6–12 weeks old) BALB/c strain mice (Specific Pathogen Free Breeding Unit, Alderley Park, Macclesfield, UK) were used. Food (SDS PCD diet; Special Diets Services Ltd, Essex, UK) and water were available *ad libitum*. The temperature was maintained at $21 \pm 2^\circ\text{C}$ and relative humidity was $55 \pm 10\%$ with a 12 h light/dark cycle.

Chemicals

TMA (97% pure) was supplied by Aldrich Chemical Co. (Gillingham, Dorset, UK). DNCB (98% pure), fluorescein isothiocyanate (FITC) isomer I (90% pure) and formalin (37% formaldehyde) were obtained from Sigma Chemical Co. (St Louis, MO, USA). TMA, DNCB and formaldehyde

were dissolved in 4:1 v/v acetone:olive oil (AOO) and FITC was formulated in 1:1 v/v acetone:dibutyl phthalate (ADBP) and were prepared freshly prior to dosing.

Sensitization

Groups of mice ($n = 5$) received 50 μL of 10% TMA, 1% DNCB or 50% formaldehyde in AOO, or 0.25% FITC in ADBP on each shaved flank. Control animals were treated concurrently with vehicle alone. Five days later this treatment was repeated. After a further 5 days, 25 μL of chemical or vehicle alone was applied to the dorsum of both ears daily for 3 consecutive days.

Preparation of cells from the draining lymph nodes

Draining (auricular) lymph nodes were excised into RPMI 1640 medium (GibcoBRL, Renfrewshire, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine (GibcoBRL), 400 $\mu\text{g}/\text{mL}$ streptomycin and 400 $\mu\text{g}/\text{mL}$ ampicillin (Sigma Chemical Co.) (RPMI-FCS) and pooled on an experimental group basis. A single cell suspension of lymph node cells (LNC; 10^7 cells/mL) was prepared under aseptic conditions by mechanical disaggregation through 200-mesh stainless-steel gauze.

Intracellular cytokine staining and flow cytometric analysis

Cells were incubated with phorbol myristate acetate (PMA; Sigma Chemical Co.) (50 ng/mL) and ionomycin (Sigma Chemical Co.; 500 ng/mL) for 3 h. Brefeldin A (1 $\mu\text{g}/\text{mL}$) (Sigma Chemical Co.) was added for the final 1.5 h. Cells (2×10^6 in 100 μL) were washed twice in FACS buffer (0.1% bovine serum albumin (BSA; Sigma Chemical Co.)/PBS/0.05% sodium azide) and incubated for 10 min (4°C) with 10 $\mu\text{g}/\text{mL}$ rat anti-mouse CD16/32 (clone 2.4G2, rat IgG2b κ ; Pharmingen, San Diego, CA, USA). Cells were washed in FACS buffer and incubated with optimal (typically 1 μg per 10^6 cells) concentrations of Tri-colour (TC)-conjugated rat monoclonal anti-mouse CD8 antibody (clone 3B5; Caltag, Burlingame, CA, USA), phycoerythrin (PE)-labelled anti-CD8 antibody (Ly-2, clone 53–6.7; Pharmingen) or FITC-labelled anti-CD4 antibody (L3T4, clone H129.19; Pharmingen) for 30 min on ice, washed, fixed in 2% paraformaldehyde in PBS and permeabilized with 0.1% saponin (Sigma Chemical Co.). Cells were incubated for 30 min at room temperature with fluorochrome-labelled anti-cytokine antibodies (anti-IFN- γ PE, clone XMG1.2; anti-IL-4 allophyco-cyanin (APC), clone 11B11; Pharmingen) or isotype controls in 0.1% saponin/FACS buffer. The following combinations of antibodies were analysed: anti-IL-4 APC with anti-CD4 FITC or anti-CD8 PE; anti-IFN- γ PE with anti-CD4 FITC or anti-CD8 TC. Labelled populations were analysed using a FACsCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) using CellQuest Pro software. An intact lymphocyte cell gate was set on cell size (forward scatter; FSC) and cell granularity (side scatter; SSC) [25]. For each sample, data from 1.5×10^5 cells were acquired and CD4⁺ IFN- γ ⁺ or IL-4⁺ and CD8⁺ IFN- γ ⁺ or IL-4⁺ cells were gated and quantified as a percentage of the intact lymphocyte pool. The frequency of positive cells in the isotype control-treated sample was subtracted from the frequency of positive cells in the cytokine-stained population.

Complement depletion of CD4⁺ and CD8⁺ T cells from lymph node cells

LNC were incubated at 2×10^7 cells/mL at 4 °C for 45 min with 4 µg/mL rat monoclonal anti-L3T4 (anti-mouse CD4; clone YTS 191.1.2, Harlan SeraLab, Crawley Down, UK), anti-Lyt.2 (anti-mouse CD8; clone YTS 169.4, Harlan SeraLab) or with rat IgG2b myeloma protein (Serotec, Kidlington, UK) diluted in RPMI-FCS. Lymphocyte populations were washed, resuspended in RPMI-FCS with 10% low toxicity rabbit complement (Harlan SeraLab) and incubated for 45 min at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells were washed and viable cell counts performed by exclusion of 0.5% trypan blue. The extent of CD4⁺ and CD8⁺ depletion was assessed by flow cytometric analysis as described above. Non-viable (propidium iodide permeable) cells were excluded from the analysis.

Culture of lymph node cells

Cells (1 mL) at 10^7 cells/mL in RPMI-FCS were cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air in the presence or absence of 2 µg/mL of concanavalin A (con A; Sigma Chemical Co.). Culture was terminated after 24 h (in the presence of con A) or after 120 h (in the absence of con A), supernatants collected and stored at -70 °C.

Cytokine determinations

The IL-4 content of culture supernatants derived from con A-stimulated LNC and IL-5, IL-10, IL-13 and IFN-γ content of supernatants derived in the absence of con A were measured by sandwich ELISAs [22]. Optical density at 450 nm was measured using an automated reader (Multiskan, Flow Laboratories, Irvine, Ayrshire, UK). A standard curve derived with murine recombinant cytokine and associated computer software (Genesis, Life Sciences International Ltd, Basingstoke, UK) were used to calculate the cytokine concentration in supernatants. Standard errors were less than 10% in most experiments.

Statistical analyses

Cytokine secretion data were evaluated using two-way ANOVA following logarithmic transformation, allowing for the replicate structure of the experimental design. The statistical significance of differences between experimental groups was calculated by comparison of individual group means using a two-sided Student's *t*-test.

Results

Animals were exposed topically to concentrations of DNCB (1%) and TMA (10%) that have been shown previously to induce equivalent levels of immunogenicity with respect to lymph node activation, measured as a function of increases in lymph node total cellularity and proliferation [22]. The frequencies of cytokine-positive CD4⁺ and CD8⁺ cells in draining lymph nodes isolated following exposure to AOO

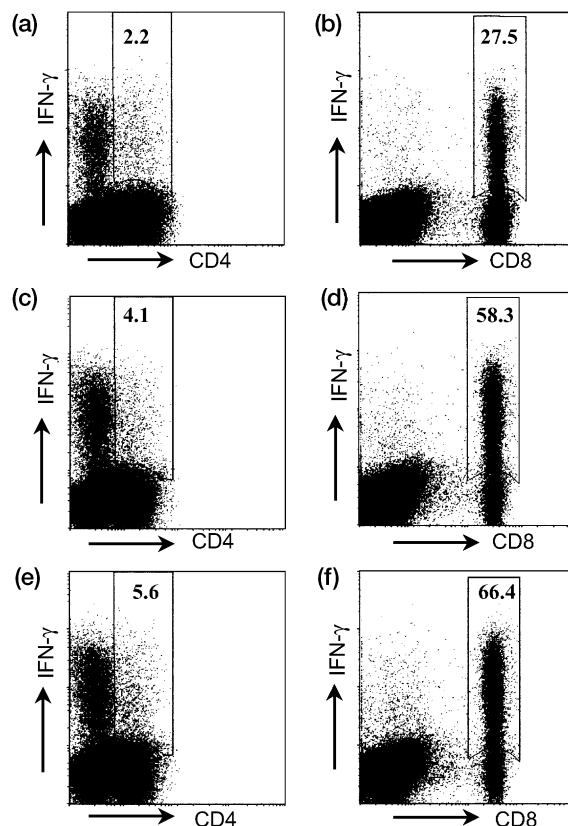


Fig. 1. IFN-γ-expressing allergen-activated lymph node cells (LNC). LNC were isolated after exposure to acetone:olive oil vehicle (a, b), 1% 2,4-dinitrochlorobenzene (c, d) or to 10% trimellitic anhydride (e, f) and stained for CD4 and CD8 and intracellular IFN-γ expression. FL1-FITC CD4⁺ vs. FL2-PE IFN-γ log dot plot (a, c, e) and a FL3-TC CD8⁺ vs. FL2-PE IFN-γ⁺ log dot plots are shown (b, d, f) for a representative experiment with percentages of cytokine-positive cells.

vehicle, to DNCB or to TMA were enumerated. The staining pattern of cytokine-positive cells is illustrated as dot plots in Figs 1 and 2 (representative experiments). The relative proportions of cytokine-expressing CD4⁺ and CD8⁺ cells have been enumerated in allergen-activated and vehicle control LNC populations (Fig. 3; mean and SE, *n* = 5 experiments). As reported previously [24], the LNC population isolated from vehicle-treated BALB/c strain mice comprised approximately 35% CD4⁺ and 15% CD8⁺ cells, and exposure to allergen was without effect on CD4⁺ and CD8⁺ cell numbers. A distinct population of CD8⁺ cells with relatively high expression of CD8 molecules was recorded in all LNC populations using both anti-CD8 PE- or -TC-conjugated antibodies. Relatively low level CD4 receptor expression was observed for all populations when measured using anti-CD4 FITC- or -TC-conjugated antibodies, although it was still possible to differentiate the CD4⁺ and CD4⁻ fractions. Between 0.7% and 3.9% CD4⁺ cells isolated from AOO-treated mice were IFN-γ⁺ compared with 6.2–27.5% of CD8⁺ cells. Topical treatment with either allergen resulted in an increased frequency of CD4⁺ and CD8⁺ IFN-γ cells. The proportion of IFN-γ⁺ CD4⁺ cells varied from 1.4% to 8.4%, and from 1.7% to 5.6%, in DNCB- and TMA-activated LNC populations, respectively. Exposure to allergen resulted in at least a twofold increase in

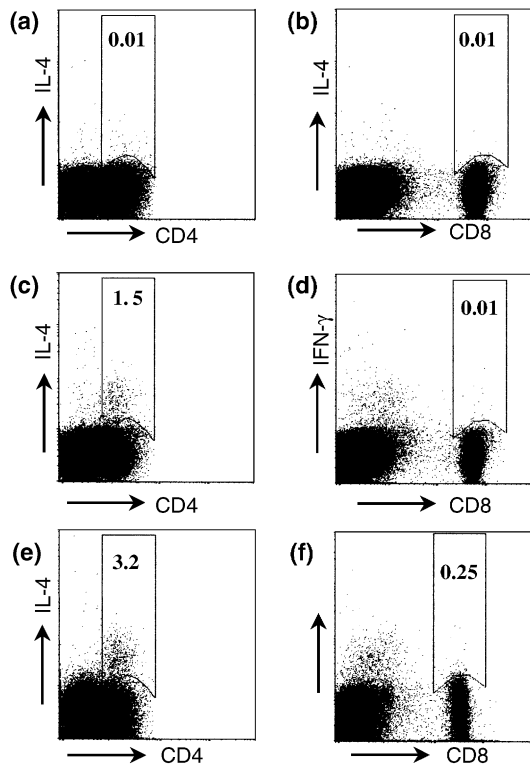


Fig. 2. IL-4-expressing allergen-activated lymph node cells (LNC). LNC were isolated after exposure to acetone:olive oil vehicle (a, b), 1% 2,4-dinitrochlorobenzene (c, d) or to 10% trimellitic anhydride (e, f) and stained for CD4 and CD8 and intracellular IL-4 expression. FL1-FITC CD4⁺ vs. FL4-APC IL-4⁺ log dot plot (a, c, e) and a FL2-PE CD8⁺ vs. FL4-APC IL-4⁺ log dot plots are shown (b, d, f) for a representative experiment with percentages of cytokine-positive cells.

IFN- γ ⁺ CD4⁺ cell numbers, with responses to DNCB somewhat more vigorous than those stimulated by TMA. High frequencies of IFN- γ ⁺ CD8⁺ cells were recorded in allergen-activated LNC populations, ranging from 12.7% to 66.4%. Similar numbers of IFN- γ ⁺ CD8⁺ cells were observed in LNC populations derived from TMA- and DNCB-exposed mice. Relatively low frequencies of IL-4⁺ T cells were detected in all LNC subsets. Less than 0.25% IL-4⁺ CD8⁺ were recorded for cells derived from vehicle- and allergen-treated animals. Similar very low numbers of IL-4⁺ CD4⁺ cells were found in LNC isolated from vehicle-exposed animals. Treatment with DNCB also resulted in the identification of relatively low numbers of IL-4⁺ CD4⁺ cells. Exposure to TMA, however, was associated with a marked up-regulation of IL-4⁺ CD4⁺ cell numbers, with an average fivefold increase in cytokine-expressing cells compared with DNCB-stimulated LNC. Comparisons of the relative proportions of type 1 and type 2 cytokine-expressing CD4⁺ cells in DNCB- and TMA-activated LNC populations revealed that the average ratio of IFN- γ ⁺ cells to IL-4⁺ cells was 28:1 (ranging from 3:1 to 70:1) and 1.2:1 (ranging from 0.7:1 to 1.8:1), respectively.

The relative contribution of CD4⁺ and CD8⁺ cells to the divergent cytokine secretion profiles stimulated by repeated application of allergen was examined by complement depletion. Populations were analysed by flow cytometry to determine percentage depletion; depleted populations routinely contained less than 5% contaminating cells. Consistent

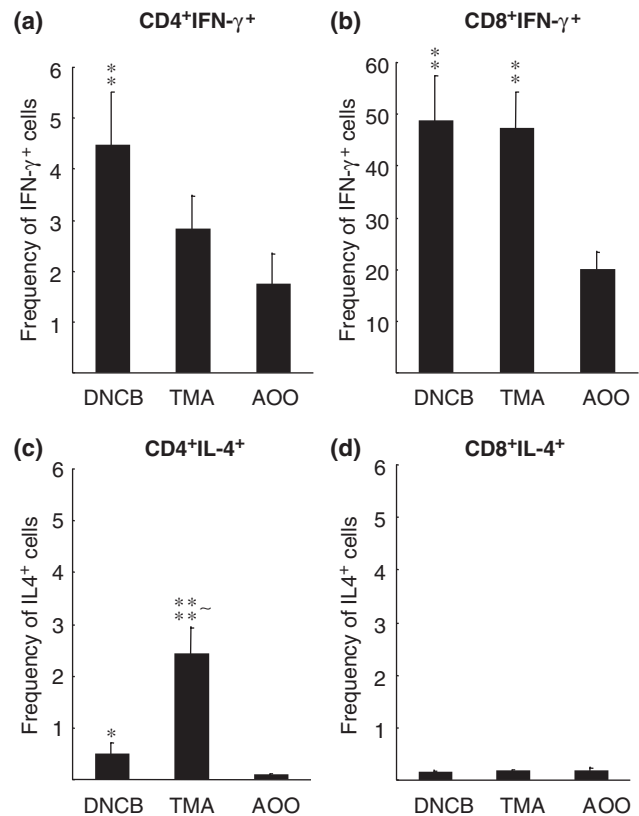


Fig. 3. Frequency of cytokine-expressing allergen-activated lymph node cells (LNC): inter-experimental variation. The frequency of IFN- γ ⁺ and IL-4⁺ CD4⁺ (a, c) and CD8⁺ (b, d) cells was enumerated in 2,4-dinitrochlorobenzene (DNCB)-, trimellitic anhydride (TMA)- and vehicle (AOO)-stimulated LNC populations and results are displayed as mean SE (for $n = 5$ experiments). * $P < 0.05$; ** $P < 0.01$: significant increase vs. vehicle-treated control populations: ** ~ $P < 0.01$: significant increase TMA- vs. DNCB-treated populations.

with previously published data [23, 24], TMA-activated LNC displayed a type 2 phenotype with high levels of mitogen-inducible IL-4 and spontaneous IL-5, IL-10 and IL-13 secretion, but relatively low levels of the type 1 cytokine IFN- γ (Fig. 4). Depletion of CD8⁺ cells had little impact on type 2 cytokine production, whereas loss of the CD4⁺ fraction abrogated expression of these cytokines. IFN- γ secretion, however, was associated with the CD8⁺ cell population. Levels of type 2 cytokines elaborated by DNCB-stimulated LNC were too low for the accurate determination of the contribution of CD4⁺ and CD8⁺ cell phenotypes (data not shown). Relatively vigorous IFN- γ expression was observed for isotype control treated DNCB-activated LNC (Fig. 5). The contribution of CD4⁺ and CD8⁺ cells to this type 1 phenotype was variable, with both subsets associated with IFN- γ secretion. In two of four experiments, depletion of CD4⁺ cells resulted in an almost complete loss of production of this cytokine, although in the remainder of the experiments, depletion of CD8⁺ cells had a greater impact on IFN- γ expression.

The impact of exposure to other allergens on the frequency of cytokine-positive cells has been investigated. Mice received topical applications of the reference allergens DNCB and TMA, or the contact allergen formaldehyde (50%) and

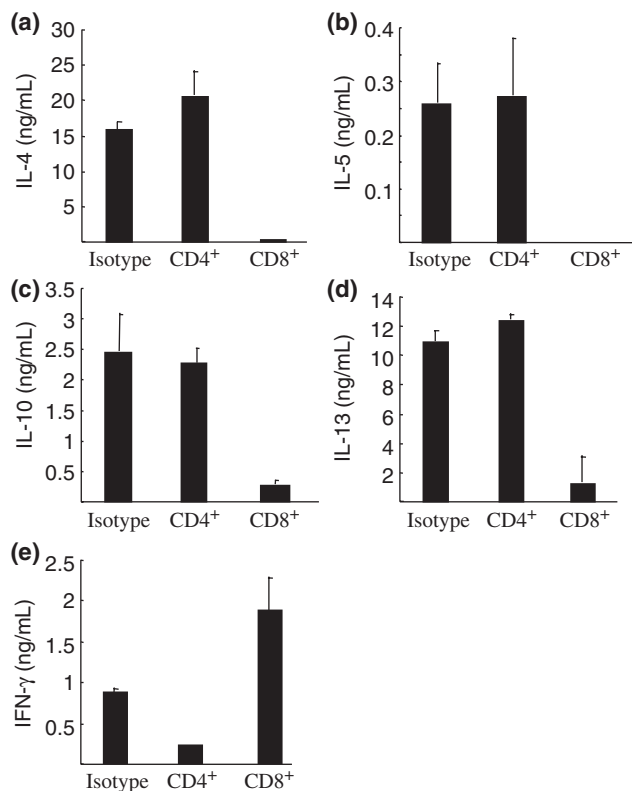


Fig. 4. Contribution of CD4 and CD8 cells to cytokine secretion phenotype of trimellitic anhydride (TMA)-activated lymph node cells (LNC). CD4⁺ (CD8 depleted), CD8⁺ (CD4 depleted) and isotype control TMA-activated LNC populations were prepared. Cells were cultured with concanavalin A (con A) for IL-4 production (a) and without con A for the production of IL-5 (b), IL-10 (c), IL-13 (d) and IFN-γ (e), measured by ELISA (mean ± SE, *n* = 3 experiments).

0.25% FITC, a sensitizing isocyanate that is associated with a type 2 phenotype [23] (Fig. 6). Exposure to both DNCB and TMA resulted in an increased frequency of CD4⁺ and CD8⁺ IFN-γ⁺ cells, but only treatment with TMA resulted in a marked elevation in IL-4⁺ CD4⁺ cells. Similar numbers of CD4⁺ and CD8⁺ IFN-γ⁺ cells were recorded after exposure to formaldehyde and FITC, but FITC treatment resulted in a very profound increase in IL-4⁺ CD4⁺ cells (approximately 10%) compared with vehicle-treated control cells.

Discussion

The data reported here suggest a complicated picture of the relative contributions of CD4⁺ and CD8⁺ T cells to the cytokine phenotype induced in mice by chemical allergens. Treatment with either class of chemical allergen resulted in an increased frequency of both CD4⁺ and CD8⁺ cells expressing IFN-γ compared with vehicle-treated controls. However, only respiratory allergen-activated LNC populations displayed a marked elevation in the number of IL-4⁺ CD4⁺ cells, with no evidence of the induction of CD8⁺ cells with type 2 phenotype. CD4⁺ cells were confirmed as the source of the preferential type 2 cytokine secretion profile induced by exposure to respiratory allergens [23, 24]. Similar results were reported by others when cytokines were measured following

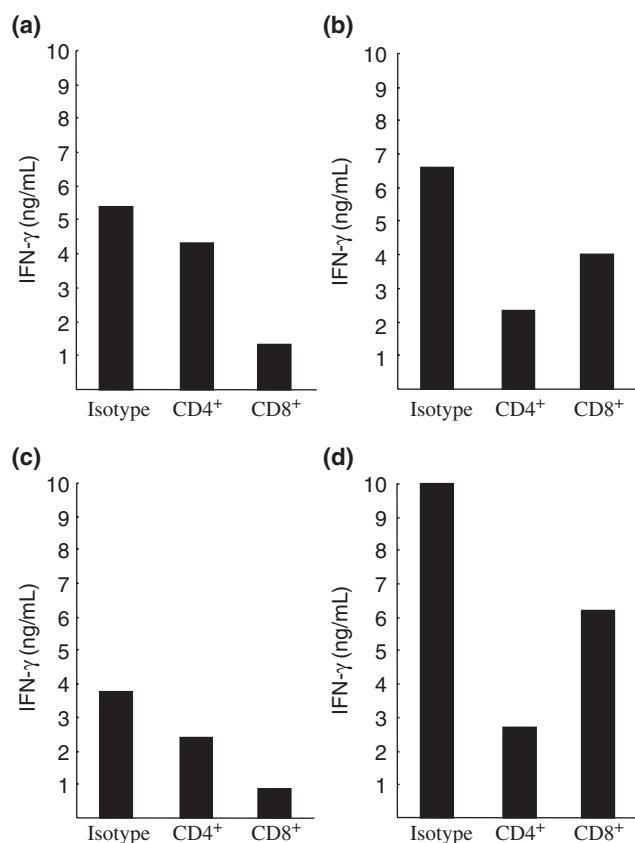


Fig. 5. Contribution of CD4 and CD8 cells to cytokine secretion phenotype of 2,4-dinitrochlorobenzene (DNCB)-activated lymph node cells (LNC). CD4⁺ (CD8 depleted), CD8⁺ (CD4 depleted) and isotype control DNCB-activated LNC populations were prepared. Cells were cultured for 120 h in the absence of concanavalin A for IFN-γ production, measured by ELISA. Mean cytokine production (ng/mL) is shown for four independent experiments (a-d).

topical exposure of BALB/c strain mice to the respiratory sensitizing diisocyanate hexamethylene diisocyanate, with type 2 cytokines expressed by CD4⁺ cells only, although IFN-γ production was a property of both subsets [26]. Although both classes of chemical allergen provoked similar increases in both CD4⁺ and CD8⁺ IFN-γ cell frequencies, only the CD8 subset contributed to the relatively low levels of this cytokine secreted by respiratory allergen-activated LNC. Both subsets contributed to the relatively large amounts of IFN-γ elaborated by contact allergen-activated LNC, despite the frequency of IFN-γ⁺ CD8⁺ cells being considerably higher (approximately 50–60%) than the IFN-γ⁺ CD4⁺ subset (approximately 5%). Thus, the induction of a type 1 phenotype with respect to increased IFN-γ cell frequency is apparently a consequence of allergen activation *per se*, but the expression of this phenotype is regulated at the level of secretion. It has been reported previously that following stimulation *in vitro* with PMA, although a similar percentage of IFN-γ-producing cells was recorded in children with atopic dermatitis compared with normal controls, levels of secreted product were significantly lower in atopics, suggesting post-translational differences in the regulation of IFN-γ production [27]. The failure of IFN-γ cells in TMA-activated LNC populations to secrete high levels of this cytokine is not

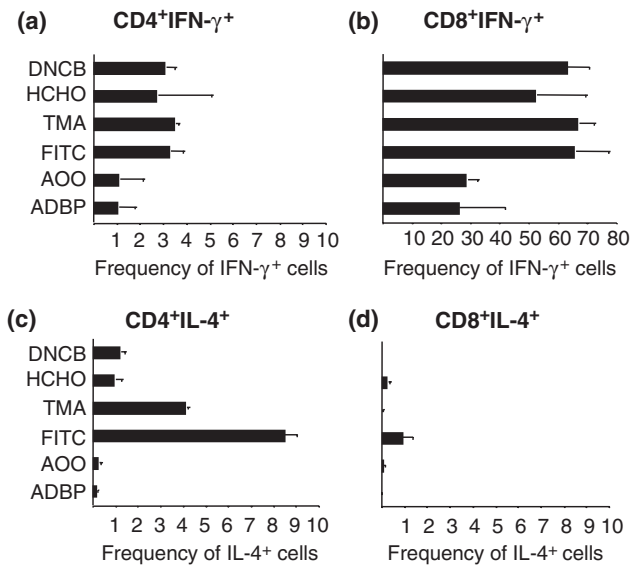


Fig. 6. Frequency of cytokine-expressing allergen-activated lymph node cells (LNC): comparisons with FITC and formaldehyde. The frequency of IFN-γ⁺ and IL-4⁺ CD4⁺ and CD8⁺ cells was enumerated in 2,4-dinitrochlorobenzene (DNCB)-, trimellitic anhydride (TMA)-, formaldehyde (HCHO)-, fluorescein isothiocyanate (FITC)- and vehicle (acetone: olive oil (AOO) or acetone: dibutyl phthalate (ADBP))-stimulated LNC populations. IFN-γ⁺ CD4⁺ (a), IFN-γ⁺ CD8⁺ (b), IL-4⁺ CD4⁺ (c) and IL-4⁺ CD8⁺ (d) cells are shown (mean and range of two independent experiments).

simply because of the fact that the high levels of type 2 cytokines expressed by this population inhibit IFN-γ secretion as depletion of type 2 cytokine-expressing CD4⁺ cells did not result in the induction of similar levels of IFN-γ to those observed following exposure to chemical contact allergens.

In order to detect intracellular cytokine, it was necessary to restimulate *in vitro* with mitogen and to block secretion with brefeldin A (data not shown). This is consistent with the experience of others using human tissue, with only low or undetectable frequencies of cytokine-positive cells detected in the absence of restimulation [18, 19]. A consequence of the requirement for restimulation is the down-regulation of CD4 expression; it has been reported previously that culture of mouse peripheral T cells with PMA/ionomycin results in the complete loss of surface CD4 for up to 24 h [28]. The extent of loss of expression of this membrane determinant in the experiments described herein did not, however, compromise detection of CD4⁺ cells. As the cells have to be restimulated with PMA/ionomycin to induce detectable intracellular cytokine expression, the important comparisons are between cells derived from quiescent lymph nodes and those derived from allergen-treated mice. Clear increases in both IL-4⁺ and IFN-γ⁺ cells were recorded following treatment with allergen; for the former, such increases were in the absence of the detection of a significant population of cytokine-positive cells in control (resting) lymph nodes. Between 0.7% and 3.9% IFN-γ⁺ CD4⁺ cells and 6.2% to 27.5% IFN-γ⁺ CD8⁺ cells were isolated from control (AOO)-treated mice. Given that such quiescent LNC populations do not secrete high levels of this cytokine spontaneously it is possible that the intracellular IFN-γ detected could be wholly the consequence of restimulation *in vitro*, or it could be that IFN-γ expression is regulated at the level of secretion [27]. Following treatment

with allergen, LNC populations are, however, sufficiently activated to secrete spontaneously type 1 or type 2 cytokines (IL-5, IL-10, IL-13 or IFN-γ) without a requirement for restimulation *in vitro*. For the expression of IL-4 by respiratory allergen activated LNC, it is necessary for LNC to be cultured in the presence of the mitogen con A. Mitogen activation *per se* does not result in IL-4 production, as LNC derived from naïve or vehicle-treated mice fail to express detectable IL-4 despite the presence of con A. The inability to detect IL-4 production in the absence of stimulation with con A may be because of reuptake of this cytokine; indeed it has been reported that the use of anti-IL-4 receptor antibodies to block reutilization enhances IL-4 detection [29].

The polarized cytokine profiles induced in mice by different classes of chemical allergen (respiratory or contact sensitizers such as TMA or DNCB, respectively) require repeated exposure to antigen to develop, reflecting the evolution of differentiated T cell function with time. Acute (3 days) treatment with allergen stimulates a mixed cytokine phenotype, with IL-4 and IFN-γ expression by both DNCB- and TMA-activated LNC. It is only after more prolonged (13 days) exposure that divergent type 1 and type 2 cytokine secretion patterns are observed [30]. A central question is what are the early events that result in the induction of these differential cytokine profiles. There is a growing appreciation that dendritic cells (DC) may play a pivotal role [31, 32]. DC display a degree of functional plasticity that can be programmed by microenvironmental conditions and, in particular, by exposure to foreign antigen. For instance, toxin from the bacterium *Bordetella pertussis* triggered DC to stimulate preferential type 1 T cell development, whereas an extract from the helminth parasite *Shistosoma mansoni* induced DC that promoted the development of type 2 cytokine-expressing T cells [33]. Such 'differentiated' DC subsets have been termed DC1 and DC2 [31, 32]. The functional polarization of DC induced by the antigenic environment in turn stimulates a polarization of T lymphocyte function. We speculate that chemical sensitizers are being misread by DC as being characteristic of a particular class of pathogenic micro-organism, and, as a consequence, inducing selective T lymphocyte responses. Indirect evidence in support of this hypothesis comes from studies that have revealed that DNCB and TMA differ with respect to the tempos of induced LC migration and DC accumulation in draining lymph nodes. Application of DNCB induces the rapid loss of LC from the skin whereas treatment with TMA results in delayed kinetics of migration of LC and subsequent accumulation in the draining lymph nodes of DC [34]. However, a general causative relationship between the dynamics of LC migration and the quality of immune response elicited by a chemical allergen remains unproven.

There are many other factors that could contribute to the selective cytokine profiles induced by DNCB and TMA. Among these are antigen dose, toxicity, solubility and skin penetration. In order to control for some of these variables, DNCB and TMA have been dosed in the same vehicle at concentrations (1% and 10%, respectively) that provoke similar levels of immunogenicity with respect to induction of equivalent levels of LNC proliferation [22]. The fact that both chemicals, in the absence of local or systemic toxicity, stimulate marked proliferative responses of similar magnitude

in the lymph node draining the site of topical exposure suggests that the divergent cytokine responses observed are independent of differences in solubility or skin penetration. Further evidence that DNCB and TMA possess intrinsic properties that stimulate divergent cytokine responses comes from studies in other rodents (Brown Norway rats) in which preferential type 1 and type 2 cytokine profiles are observed, respectively [22]. Antigen dose may be more relevant for understanding the mechanisms of chemical-allergen-induced cytokine skewing. It has been reported previously that for a single protein antigen, dose, in the context of antigen load on the antigen-presenting cell, can have a profound impact on polarization of cytokine responses [35]. In these experiments, priming with high doses of antigen lead to type 1 cytokine expression whereas low dose antigen preferentially induced type 2 cells.

The observation that exposure of mice to chemical respiratory allergens induces a marked increase in IL-4⁺ CD4⁺ cells, but not in IL-4⁺ CD8⁺ cells, is consistent with the experience of many investigators that have examined the cellular provenance of type 2 cytokines induced in patients with asthma or atopy to protein allergens [15, 16, 18, 36]. There are other reports, however, that have identified both CD4⁺ and CD8 cells as potential sources of type 2 cytokines [19]. The reasons for the conflicting observations regarding type 2 cytokine-expressing cells are unclear, but may relate to the disease status (exacerbated vs. stable state), source of cells (peripheral blood vs. bronchoalveolar lavage), polyclonal vs. specific stimulus of cells or the age of subjects. In sequential studies using the same donors it has been reported for example that concurrent viral infection influences type 1/type 2 cytokine expression patterns [37]. An IFN- γ -secreting CD8⁺ cell population has also been observed in the peripheral blood of asthmatic subjects [18, 19, 36]. Similar studies have not been conducted with tissue derived from individuals with respiratory sensitization to low-molecular-weight chemicals, although it has been reported recently that the majority of T cell clones generated from the peripheral blood of isocyanate-sensitized workers were IFN- γ -secreting CD8⁺ cells [20]. It has been speculated that this population may play an immunoregulatory role, suppressing eosinophilic and IgE-mediated inflammatory reactions. Other studies have demonstrated that chemokine production, particularly the expression of monocyte-chemoattractant protein-1 that has histamine-releasing properties, by peripheral mononuclear cells may play a role in the pathogenesis of occupational asthma to diisocyanates [38, 39].

In summary, these data demonstrate that the divergent immune responses induced in mice by different classes of chemical allergen are independent of changes in the frequency of IFN- γ ⁺ cells, but are associated with an increased frequency of IL-4-expressing CD4⁺ Th cells in respiratory allergen-activated LNC populations. The vigorous IFN- γ production by contact allergen-activated LNC is likely to be regulated at the level of protein secretion.

References

- Sastre J, Vandenplas O, Park HS. Pathogenesis of occupational asthma. *Eur Respir J* 2003; 22:364–73.
- Wild LG, Lopez M. Occupational asthma caused by high-molecular-weight substances. *Immunol Allergy Clin North Am* 200; 23:235–50.
- Frew AJ. What can we learn about asthma from studying occupational asthma? *Ann Allergy Asthma Immunol* 2003; 90:7–10.
- Topping MD, Venables KM, Luczynska CM, Howe W, Newman Taylor AJ. Specificity of the human IgE response to inhaled acid anhydrides. *J Allergy Clin Immunol* 1986; 77:834–42.
- Czuppon AB, Merget R, Raulf-Heimsoth M, Bruening T. Comparison of IgE antibody concentrations to pyromellitic dianhydride-modified laminin and human serum albumin in sera of exposed workers with respiratory complaints. *Int Arch Allergy Immunol* 2002; 127:79–81.
- Tee RD, Cullinan P, Welch J, Burge PS, Newman Taylor AJ. Specific IgE antibody to isocyanates: a useful diagnostic role in occupational asthma. *J Allergy Clin Immunol* 1998; 101: 709–15.
- Wass U, Belin L. Immunological specificity of isocyanate-induced IgE antibodies in serum from 10 sensitized workers. *J Allergy Clin Immunol* 1989; 83:126–35.
- Mapp C, Boschetto P, Miotto D, De Rosa E, Fabbri LM. Mechanisms of occupational asthma. *Ann Allergy Asthma Immunol* 1999; 83:645–64.
- Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 2003; 111:450–63.
- Bentley AM, Maestrelli P, Saetta M et al. Activated T lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. *J Allergy Clin Immunol* 1992; 89:821–9.
- Del Prete GF, De Carli M, D'Elia MM et al. Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol* 1993; 23:1445–9.
- Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions of mouse CD4⁺ cells. *Adv Immunol* 1989; 46:111–47.
- Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8⁺ T cells into cytotoxic CD8⁺ T cells secreting Th1 or Th2 cytokines. *Immunity* 1995; 2:271–9.
- Durham SR, Till SJ, Corrigan CJ. T lymphocytes in asthma: bronchial vs. peripheral responses. *J Allergy Clin Immunol* 2000; 106:S221–6.
- Ying S, Humbert M, Barkans J et al. Expression of IL-4 and IL-5 mRNA and protein product by CD4⁺ and CD8⁺ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* 1997; 158:3539–44.
- Till S, Dickason R, Huston D et al. IL-5 secretion by allergen-stimulated CD4⁺ T cells in primary culture: relationship to expression of allergic disease. *J Allergy Clin Immunol* 1997; 99:563–9.
- O'Brien RM, Xu H, Rolland JM, Byron KA, Thomas WR. Allergen-specific production of interferon-gamma by peripheral blood mononuclear cells and CD8 T cells in allergic disease and following immunotherapy. *Clin Exp Allergy* 2000; 30:333–40.
- Magnan AO, Mely LG, Camilla CA et al. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. *Am J Respir Crit Care Med* 2000; 161:1790–6.
- Cho SH, Stanciu LA, Begishvili T, Bates PJ, Holgate ST, Johnston SL. Peripheral blood CD4⁺ and CD8⁺ T cell type 1 and type 2 cytokine production in atopic asthmatic and normal subjects. *Clin Exp Allergy* 2002; 32:427–33.
- Maestrelli P, Del Prete GF, De Carli M et al. CD8 T-cell clones producing interleukin-5 and interferon-gamma in bronchial mucosa of patients with asthma induced by toluene diisocyanate. *Scand J Work Environ Health* 1994; 20:376–81.

- 21 Zeiss CR, Patterson R, Pruzansky JJ, Miller M, Rosenberg M, Levitz D. Trimellitic anhydride-induced airway syndromes: clinical and immunologic studies. *J Allergy Clin Immunol* 1977; 60:96–103.
- 22 Dearman RJ, Warbrick EV, Skinner R, Kimber I. Cytokine fingerprinting of chemical allergens: species comparisons and statistical analyses. *Food Chem Toxicol* 2002; 40: 1881–92.
- 23 Dearman RJ, Kimber I. Role of CD4⁺ T helper 2-type cells in cutaneous inflammatory responses induced by fluorescein isothiocyanate. *Immunology* 2000; 101:442–51.
- 24 Dearman RJ, Moussavi A, Kemeny DM, Kimber I. Contribution of CD4⁺ and CD8⁺ T lymphocyte subsets to the cytokine secretion patterns induced in mice during sensitization to contact and respiratory chemical allergens. *Immunology* 1996; 89:502–10.
- 25 Chrest FJ, Buchholz MA, Kim YH, Kwon TK, Nordin AA. Identification and quantification of apoptotic cells following anti-CD3 activation of murine G0 T cells. *Cytometry* 1993; 14:883–90.
- 26 Herrick CA, Das J, Xu L, Wisniewski AV, Redlich CA, Bottomly K. Differential roles for CD4 and CD8T cells after diisocyanate sensitization: genetic control of TH2-induced lung inflammation. *J Allergy Clin Immunol* 2003; 111:1087–94.
- 27 Tang M, Kemp A. Production and secretion of interferon-gamma (IFN-gamma) in children with atopic dermatitis. *Clin Exp Immunol* 1994; 95:66–72.
- 28 Anderson SJ, Coleclough C. Regulation of CD4 and CD8 expression on mouse T cells. Active removal from the cell surface by two mechanisms. *J Immunol* 1993; 151:5123–34.
- 29 Bancroft AJ, McKenzie ANJ, Grencis R. A critical role for IL-13 in resistance to intestinal nematode infection. *J Immunol* 1998; 160:3453–61.
- 30 Moussavi A, Dearman RJ, Kimber I, Kemeny DM. Cytokine production by CD4⁺ and CD8⁺ T cells in mice following primary exposure to chemical allergens: evidence for functional differentiation of T lymphocytes in vivo. *Int Arch Allergy Immunol* 1998; 116:116–23.
- 31 Kapsenberg ML, Hilkens CMU, Wierenga EA, Kalinski P. The role of antigen-presenting cells in the regulation of specific T cell responses. *Curr Opin Immunol* 1998; 10:607–13.
- 32 Manickasingham SP, Edwards AD, Schulz O, Reis e Sousa C. The ability of murine dendritic cell subsets to direct T helper cell differentiation is dependent on microbial signals. *Eur J Immunol* 2003; 33:101–7.
- 33 de Jong EC, Vieira PL, Kalinski P et al. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell polarizing signals. *J Immunol* 2002; 168:1704–9.
- 34 Cumberbatch M, Dearman RJ, Griffiths CEM, Kimber I. Epidermal Langerhans cell migration and sensitisation to chemical allergens. *APMIS* 2003; 111:797–804.
- 35 Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4⁺ T cells. *J Exp Med* 1995; 182:1591–6.
- 36 Brightling CE, Symon FA, Birring SS, Bradding P, Pavord ID, Wardlaw AJ. TH2 cytokine expression in bronchoalveolar lavage fluid T lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis. *J Allergy Clin Immunol* 2002; 110:899–905.
- 37 Barth H, Berg PA, Klein R. Methods for the in vitro determination of an individual disposition towards Th1- or Th2-reactivity by the application of appropriate stimulatory antigens. *Clin Exp Immunol* 2003; 134:78–85.
- 38 Lummus ZL, Alam R, Bernstein JA, Bernstein DI. Diisocyanate antigen-enhanced production of monocyte chemoattractant protein-1, IL-8, and tumor necrosis factor-alpha by peripheral mononuclear cells of workers with occupational asthma. *J Allergy Clin Immunol* 1998; 102:265–74.
- 39 Bernstein DI, Cartier A, Cote J et al. Diisocyanate antigen-stimulated monocyte chemoattractant protein-1 synthesis has greater test efficiency than specific antibodies for identification of diisocyanate asthma. *Am J Respir Crit Care Med* 2002; 166: 445–50.