

Cytokine profiling of chemical allergens in mice: impact of mitogen on selectivity of response

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ABSTRACT: A major constraint in the development of methods for the identification of chemical respiratory allergens is the continuing uncertainty regarding the mechanisms of this disease and in particular the role of IgE antibody. There is, however, increasing evidence that respiratory sensitization is favoured by the induction of a selective type 2 cytokine response. The current investigations focus on the potential application of cytokine profiling to the identification of chemical respiratory sensitizers. The objective is to determine the optimal configuration of the method for discrimination between chemical contact and respiratory sensitizers. The reference contact sensitizer 2,4-dinitrochlorobenzene (DNCB) and reference respiratory sensitizer trimellitic anhydride (TMA), which have been shown to induce type 1 and type 2 cytokine profiles, respectively, were utilized. Variables investigated included cell concentration, time in culture, dosing regimens (a 13 day and a truncated 8 day protocol) and the impact of restimulation *in vitro* with T cell mitogens. Cell culture conditions were critical for the selectivity of the response, with the addition of mitogen resulting in a less discriminatory pattern of cytokine expression, particularly for the type 1 cytokine interferon γ (IFN- γ). Furthermore, a 13 day exposure period was required for vigorous expression of IFN- γ by DNCB-activated cells, whereas type 2 cytokine expression by TMA-stimulated cells was recorded after 8 days. These data demonstrate that the most optimal method for cytokine profiling is a chronic (13 day) exposure regime followed by culture of lymph node cells at 10^7 cells ml^{-1} for 120 h in the absence of mitogen. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: chemical allergens; cytokines; mitogen; trimellitic anhydride; 2,4-dinitrochlorobenzene

Introduction

Allergic hypersensitivity of the respiratory tract is an important occupational health problem. However, despite considerable research effort in this area over the last decade, there are currently no well-validated or widely accepted test methods for the prospective identification of chemical respiratory sensitizers (Arts and Kuper, 2007; Boverhof *et al.*, 2008; Holsapple *et al.*, 2006). Potential approaches for the identification of potential respiratory sensitizers encompass those that seek to mimic the clinical manifestations of the disease (the elicitation phase). These include guinea pig, mice and rat models that utilize various routes of exposure for sensitization and monitor challenge-induced changes in respiratory function as a read-out for allergenicity (Farraj *et al.*, 2004; Karol, 1991; Pauluhn, 2003; Rattray *et al.*, 1994; Zhang *et al.*, 2006). The measurement of such changes is technically demanding, few chemicals have been examined to date and there is a desire to develop methods that are based on the induction or sensitization phase of the response and that could represent a reduction and/or refinement of animal usage. A major impediment to the development of such methods has been continuing uncertainty regarding the relevant immunobiological mechanisms. Whereas protein allergy is generally acknowledged to have an IgE-dependent mechanism, a universal role for IgE in the pathogenesis of chemical respiratory allergy remains controversial (Frew, 1996; Isola *et al.*, 2008; Jones *et al.*, 2006). There is less controversy regarding the potential role of the selective development of type 2 immune responses (Frew, 1996; Jones *et al.*, 2006; Isola *et al.*, 2008; Kimber and Dearman, 1997; Mapp *et al.*, 1999). Not only is the type 2 cytokine interleukin (IL)-4 required for IgE antibody responses, but IL-4 and IL-10 are mast cell growth and

differentiation factors, IL-5 is a key cytokine in the development and recruitment of eosinophils and IL-13 plays an important role in the hypersecretion of mucus, all of which are important in the development of asthma (Kay, 2006). It has been demonstrated that, in individuals with chemical respiratory allergy, eosinophils and T lymphocytes are recruited into the site of inflammation (Bentley *et al.*, 1992; Jones *et al.*, 2006), and such lymphocytes selectively express type 2 cytokines (Del Prete *et al.*, 1993; Jones *et al.*, 2006; Maestrelli *et al.*, 1994).

Various methods based on the assessment of allergen-induced cytokine expression patterns for the identification of chemical respiratory sensitizers have been investigated. These include methods in which cytokine expression is measured in rats (Dearman *et al.*, 2002b; Kouadio *et al.*, 2005; Pauluhn, 2008) or in mice (Dearman *et al.*, 2002b; Hayashi *et al.*, 2001; Manetz *et al.*, 2001; Plitnick *et al.*, 2002, 2005), at the level of mRNA (Betts *et al.*, 2002; Hayashi *et al.*, 2001; Manetz *et al.*, 2001; Plitnick *et al.*, 2002, 2005; Ryan *et al.*, 1998; Vandebriel *et al.*, 2000; Warbrick *et al.*, 1998) or at the level of protein secretion (Dearman *et al.*, 2002a,b; Matheson *et al.*, 2001; Vandebriel *et al.*, 2000). Although the inhalation route of exposure has been used (Farraj *et al.*, 2004;

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2006; Kim *et al.*, 2006; Kouadio *et al.*, 2005), the dermal route of exposure has been utilized more extensively, chosen to facilitate analyses of cytokine responses induced in discrete draining lymph nodes (Dearman *et al.*, 2002a; Dearman and Kimber, 2001; Vandebriel *et al.*, 2000). Given that the purpose of these experiments is not to replicate aspects of the clinical symptoms of the disease, but to provoke the quality of immune response that promotes the subsequent development of immediate type hypersensitivity responses, the dermal route is appropriate. Indeed, regardless of whether sensitization to chemical respiratory allergens is achieved through exposure to the skin or the respiratory tract, similar patterns of antibody isotypes and cytokines are induced in BALB/c strain mice (Ban *et al.*, 2006; Dearman *et al.*, 1991; Matheson *et al.*, 2001). There is also some clinical evidence (Nemery and Lenaerts, 1993) and experimental data to indicate that clinical symptoms may be provoked following skin contact (Ban *et al.*, 2006; Kimber and Dearman, 2002; Matheson *et al.*, 2001).

Other important experimental variables include the dosing regimen, with 8 day (Vandebriel *et al.*, 2000) and 13 day exposure protocols used (Dearman *et al.*, 2002a,b), and the culture conditions for cytokine generation. The use of T cell mitogens such as concanavalin A (con A) and anti-cluster of differentiation (CD)3 antibody has been proposed (Ulrich *et al.*, 2001; Vandebriel *et al.*, 2000, 2003) to enhance the sensitivity of the response and to reduce the number of cells required. One of the issues that will be addressed herein is whether this increase in sensitivity may be at the loss of selectivity of the cytokine secretion profile. Previously we have examined cytokine secretion profiles measured after culture of draining lymph node cells (LNC) in the absence of further restimulation *in vitro* (Dearman *et al.*, 2002a,b, 2003). We have demonstrated that topical exposure of BALB/c strain mice over a 13 day period to the reference contact allergen 2,4-dinitrochlorobenzene (DNCB) and the reference respiratory allergen trimellitic anhydride (TMA) results in the preferential expression of type 1 and type 2 cytokines, respectively. In the current studies we have examined the effect of culture conditions (presence of mitogen, cell concentration and time in culture) and exposure regimen on the cytokine secretion profiles provoked by the reference allergens DNCB and TMA, in order to determine the optimal protocol for differentiating between contact and respiratory allergens. In these experiments, cytokines have been measured by cytokine array technology (Luminex) rather than sandwich enzyme-linked immunosorbent assay (ELISA) as conducted previously (Dearman *et al.*, 2002a, b, 2003). This method allows for the measurement of multiple cytokines in a small sample volume, hence providing for the possibility of a reduction in the number of animals required per test group.

Methods

Mice

Young adult (6–12 weeks old) female BALB/c strain mice (Harlan Seralab, Bicester, Oxfordshire, UK) were used throughout these studies. Mice were housed in metal cages and food (SDS PCD pelleted diet; Special Diets Services Ltd, Witham, UK) and water were available *ad libitum*. The ambient temperature was maintained at $21 \pm 2^\circ\text{C}$ and relative humidity was $55 \pm 10\%$ with a 12 h light/dark cycle. All experiments were carried out under the provisions of the Animals (Scientific Procedures) Act, 1986.

Chemicals

2,4-Dinitrochlorobenzene (98% pure) and trimellitic anhydride (97% pure) were obtained from Sigma Chemical Co (St Louis, MO, USA). TMA was stored under anhydrous conditions. TMA and DNCB were dissolved in 4 : 1 acetone:olive oil (AOO). Solutions were prepared freshly immediately prior to dosing.

Sensitization of Mice for Lymph Node Cell Preparation

Chronic exposure protocol. A 13 day exposure protocol was utilized as reported previously (Dearman *et al.*, 2003). Groups of mice ($n = 5$ or 10) received 50 μl of 10% TMA in AOO or 1% DNCB in AOO or AOO vehicle alone bilaterally on each shaved flank. Five days later this treatment was repeated. After a further 5 days, 25 μl of chemical was applied to the dorsum of both ears daily for three consecutive days.

Short exposure protocol. In some experiments an 8 day exposure protocol was utilized as reported previously (Vandebriel *et al.*, 2000). Groups of mice ($n = 5$ or 10) received 25 μl of 10% TMA in AOO or 1% DNCB in AOO or AOO vehicle alone on the dorsum of both ears daily for three consecutive days.

Preparation and Culture of Cells from Draining Lymph Nodes

Eight or 13 days after the initiation of treatment, draining auricular lymph nodes were excised and pooled for each experimental group. A single cell suspension of LNC was prepared under aseptic conditions by gentle mechanical disaggregation through sterile 200-mesh stainless steel gauze. Viable cell counts were performed by exclusion of 0.5% trypan blue and total cellularity per lymph node recorded. LNC were cultured in RPMI-1640 growth medium (Gibco, Renfrewshire, UK) supplemented with 25 mM HEPES, 400 $\mu\text{g ml}^{-1}$ streptomycin, 400 $\mu\text{g ml}^{-1}$ ampicillin and 10% heat-inactivated fetal calf serum (RPMI-FCS). Cells (1 ml aliquots) at 10^7 cells ml^{-1} or 10^6 cells ml^{-1} in RPMI-FCS were seeded into 24-well tissue culture plates and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air in the presence or absence of 5 or 2 $\mu\text{g ml}^{-1}$ of con A (Sigma). For restimulation with anti-CD3 antibody (clone 145-2C11; BD Pharmingen, San Diego, CA, USA), plates were pre-coated overnight at 4°C with 5 $\mu\text{g ml}^{-1}$ of antibody diluted in sodium carbonate buffer, pH 9.6. Culture was terminated after 24–120 h, supernatants collected, centrifuged at 100g for 5 min and stored at -70°C prior to analysis. For the assessment of lymphocyte proliferation, additional aliquots of LNC were seeded in quintuplicate into 96-well microtitre plates (2.5×10^5 cells well^{-1}) and cultured for 24 h in the presence of 2 μCi [^3H]-methyl thymidine ([^3H]-TdR; specific activity 2 Ci mmol^{-1} ; Amersham International, Amersham, UK). Culture was terminated by cell harvesting and incorporation of ^3H -TdR was measured by β -scintillation counting.

Cytokine Determinations by Cytokine Array

Concentrations of IL-4, IL-5, IL-10, IL-13 and interferon γ (IFN- γ) were measured in supernatants using the Bio-Plex™ cytokine array system according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). A customized set of fluorescent anti-cytokine multiplex microbeads was supplied together with recombinant standards for the generation of a standard curve for each cytokine and the assay was performed in a 96-well

microtitre plate format. The wells of the filter plate were prewet with 100 μ l of Bio-plex assay buffer which was then removed by vacuum filtration. Multiplex beads (50 μ l per well) were added and the buffer removed by vacuum filtration. Serial (1 in 4) dilutions of the recombinant cytokines were prepared (1.95–32 000 pg ml⁻¹) in Bio-Plex standard diluent A. Diluted standard or test sample (50 μ l) was added and the sealed plate incubated at room temperature for 30 s (shaken at 1100 rpm) followed by a further 30 min (shaken at 300 rpm). Detection antibody (25 μ l) was added to each well and the sealed plate incubated at room temperature for 30 s (shaken at 1100 rpm) followed by a further 30 min (shaken at 300 rpm). Streptavidin-phycoerythrin (50 μ l) was added to each well and the sealed plate incubated at room temperature for 30 s (shaken at 1100 rpm) followed by a further 10 min (shaken at 300 rpm). Between each step the plates were washed one to three times with Bio-Plex wash buffer and the buffer removed by vacuum filtration. The beads were resuspended in 125 μ l of Bio-Plex assay buffer, the plate was sealed and shaken at 1100 rpm for 30 s at room temperature immediately prior to analysis on a Luminex 100 multiple analyte profiler (MiraiBio Hitachi Genetic Systems, Alameda, USA).

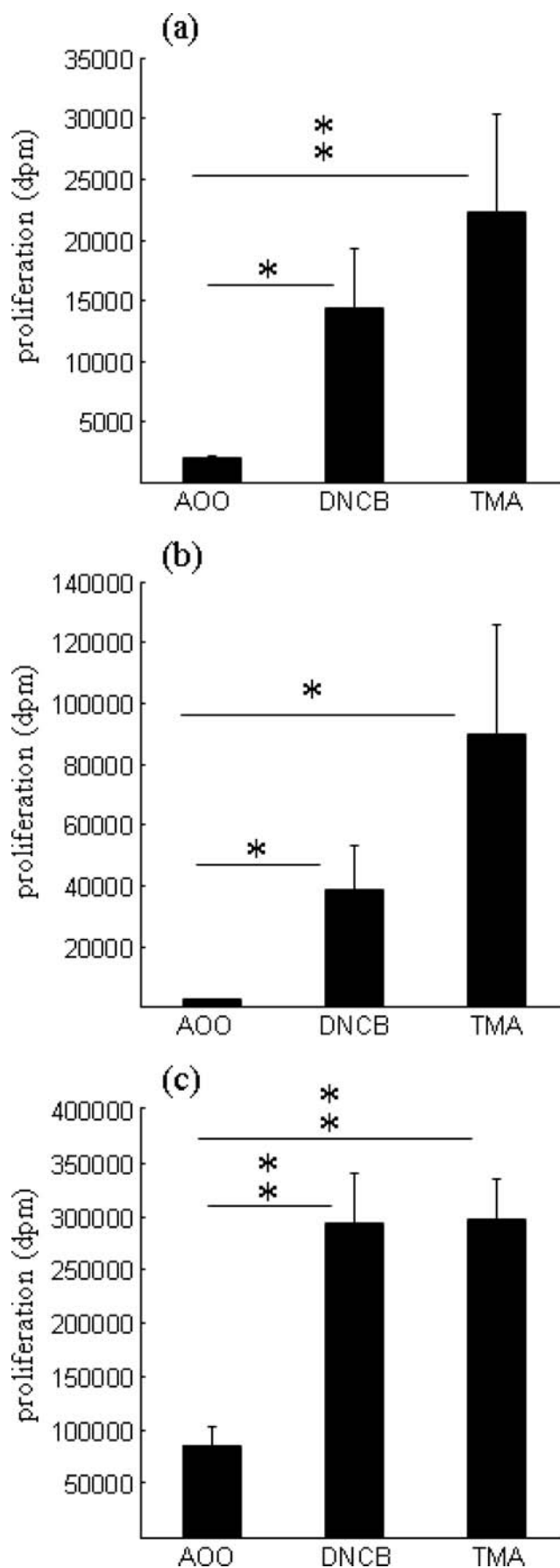
Statistical Analyses

Proliferation data from LNC preparations were considered by analysis of variance allowing for experiment, treatment, medium control and treatment by medium control interaction. Data were log transformed to stabilize the variance. Cytokine secretion data were evaluated using two-way analysis of variance 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 based on the error mean square of the analysis of variance.

Results

BALB/c strain mice were exposed topically to 1% DNCB, 10% TMA or to AOO vehicle alone. Thirteen days after the initiation of exposure, draining auricular lymph nodes were excised and a single cell suspension of LNC prepared. As observed previously (Dearman *et al.*, 2002b, 2008), treatment with chemical allergen resulted in marked lymph node activation, with approximately five-fold increases in cellularity compared with vehicle-treated controls. Thymidine incorporation (24 h) was measured for cells cultured in medium alone, in the presence of the T cell mitogen con A (5 μ g ml⁻¹) or in tissue culture plates pre-coated with anti-CD3 antibody and 24 h thymidine incorporation measured (Fig. 1). In the absence of restimulation *in vitro*, relatively low

Figure 1. Proliferation of allergen-activated and control lymph node cells: impact of mitogen. BALB/c strain mice were exposed topically to 1% DNCB or to 10% TMA, or to AOO vehicle alone ($n = 5$ for chemical allergen; $n = 10$ for AOO). Thirteen days after the initiation of exposure, draining lymph nodes were excised, pooled on a treatment group basis and a single cell suspension prepared. Cells were cultured at 2.5×10^6 cells ml⁻¹ in the presence of medium alone (a), on plates pre-coated with 5 μ g ml⁻¹ of anti-CD3 antibody (b) or in the presence of 5 μ g ml⁻¹ of con A (c). Cells were cultured for 24 h with ³H-TdR prior to automated harvesting. Results are shown as mean proliferative responses (dpm) and SE of $n =$ three to five independent experiments. Statistical significance of differences between treatment groups was analysed by analysis of variance following logarithmic transformation, * $P < 0.05$; ** $P < 0.01$.



levels of thymidine incorporation were recorded for LNC from AOO-treated animals whereas significantly higher levels of spontaneous proliferation ($p < 0.05$) were observed for cells isolated from DNCB- and TMA-exposed mice. Restimulation with anti-CD3 was without marked impact on thymidine incorporation by cells from AOO-exposed mice, whereas significant increases in proliferation were recorded for allergen-treated mice ($p < 0.05$). The mitogen con A stimulated significantly higher levels of proliferation than did anti-CD3 treatment, with very vigorous

responses recorded for cells from AOO-treated mice (mean 85 332 disintegrations per minute, dpm) and levels of 292 340 dpm and 296 623 dpm achieved for LNC isolated from DNCB- and TMA-exposed animals.

The impact of restimulation with con A, cell concentration and period of culture on the cytokine secretion profile of LNC isolated after chronic (13 day) exposure was examined (Fig. 2). Cells were cultured in the presence and absence of $5 \mu\text{g ml}^{-1}$ con A, at 10^6 cells ml^{-1} or at 10^7 cells ml^{-1} and for 24 or 120 h. In the absence

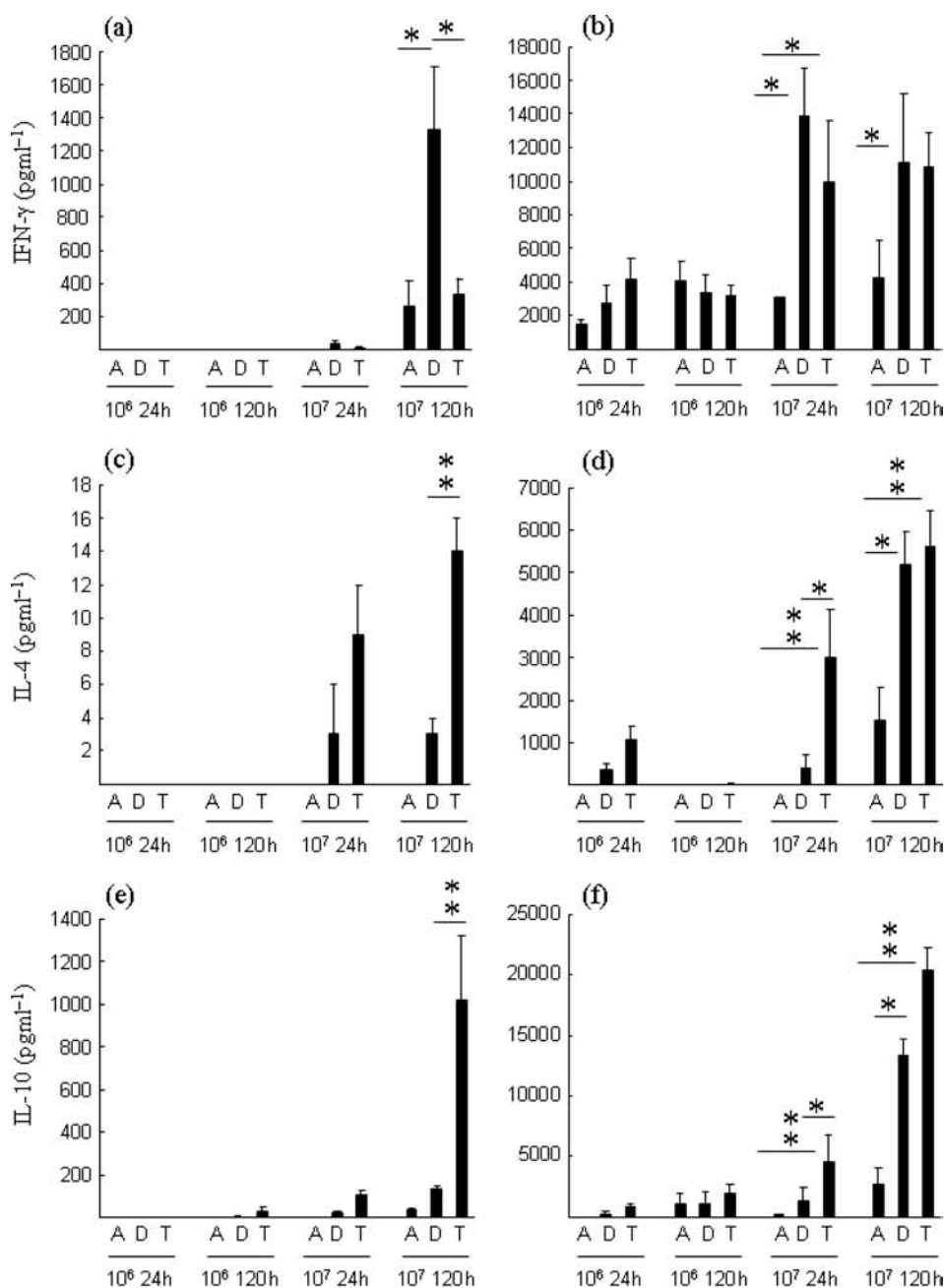


Figure 2. Cytokine expression profiles of allergen-activated lymph node cells: effect of cell concentration and mitogen. BALB/c strain mice were exposed topically to 1% DNCB (D) or to 10% TMA (T), or to AOO vehicle alone (A) ($n = 5$ for chemical allergen; $n = 10$ for AOO). Thirteen days after the initiation of exposure, draining lymph nodes were excised, pooled on a treatment group basis and a single cell suspension prepared. Cells were cultured at 10^6 or 10^7 cells ml^{-1} in the presence of medium alone (a, c and e) or in the presence of $5 \mu\text{g ml}^{-1}$ of con A (b, d and f). Cells were cultured for 24 or 120 h and culture supernatants isolated. Cytokine concentration was determined by cytokine bead microarray (a and b, IFN- γ ; c and d, IL-4; e and f, IL-10). Data are shown as mean and SE pg/ml for three to five independent experiments. Statistical significance of differences between treatment groups was analysed by analysis of variance following logarithmic transformation, * $P < 0.05$; ** $P < 0.01$.

of restimulation *in vitro*, cells cultured at 10^6 cells ml^{-1} for 24 h failed to elaborate detectable levels of cytokines (<10 pg ml^{-1}); even after 120 h of culture, levels of both IFN- γ and IL-4 remained below the lower limits of detection and only very low levels of IL-10 (<50 pg ml^{-1}) were detected in supernatants derived from LNC isolated from DNCB- and TMA-treated mice. Higher levels of cytokine were recorded in supernatants from LNC cultured at 10^7 cells ml^{-1} . At the earlier (24 h) time point, allergen-induced IL-4 and IL-10 expression reached detectable levels, with somewhat more vigorous expression by TMA- compared with DNCB-activated LNC, although these did not reach statistical significance. After 120 h of culture there was marked allergen-induced cytokine expression with preferential induction of the type 1 cytokine IFN- γ induced by DNCB and selective expression of the type 2 cytokines IL-4 and IL-10 provoked by exposure to TMA (Fig. 2). Restimulation *in vitro* with the mitogen con A resulted in a very marked increase in expression of all cytokines by both allergen-activated LNC and LNC derived from vehicle-treated animals (Fig. 2). This was particularly apparent for the type 1 cytokine IFN- γ , such that LNC isolated from AOO-treated animals cultured at 10^6 cells ml^{-1} for 24 h in the presence of con A secreted similar amounts of this cytokine to those observed following culture of 10^7 cells ml^{-1} DNCB-activated LNC for 120 h. There was no significant difference between LNC derived from vehicle- and allergen-treated mice with respect to IFN- γ production for cells cultured for 24 h or 120 h at 10^6 cells ml^{-1} in the presence of con A. At the higher cell concentration (10^7 cells ml^{-1}), significantly higher levels of this cytokine were expressed by allergen-activated LNC and those derived from control (vehicle-treated) animals. However, there was no difference between levels produced by DNCB- and TMA-activated LNC. The selectivity of cytokine secretion profiles induced by DNCB and TMA induced was maintained for the type 2 cytokines IL-4 and IL-10 when cells were cultured with con A at both cell concentrations for 24 h, with significantly higher levels provoked in each case by TMA- compared with DNCB-stimulated LNC. At the later time point (120 h), similar levels were recorded for DNCB- and TMA-activated cells. A similar pattern of cytokine responses was observed for the type 2 cytokines IL-5 and IL-13, and the concentration of con A (2 or 5 μg ml^{-1}) was without major impact (data not shown).

Given the loss of selectivity for the type 1 cytokine IFN- γ following restimulation with con A, the influence of an alternative mitogen (anti-CD3 antibody) on cytokine expression was examined. Cells were isolated from draining lymph nodes 13 days after the initiation of exposure to chemical allergen or to vehicle alone and cultured at 10^6 cells ml^{-1} for 24 h in tissue culture plates pre-coated with anti-CD3 antibody according to the method of Ulrich *et al.* (2001). Expression of IFN- γ , IL-4, IL-5, IL-10 and IL-13 was measured in two independent experiments (Fig. 3). LNC derived from AOO-treated mice expressed undetectable levels of cytokines, with the exception of the type 2 cytokine IL-5. Restimulation of allergen-activated LNC with anti-CD3 antibody resulted in detectable levels of all cytokines analysed. However, treatment with TMA and DNCB resulted in similar levels of IFN- γ expression (and at lower levels than those achieved following restimulation with con A), whereas considerably higher levels of type 2 cytokines (IL-4, IL-5, IL-10 and IL-13) were recorded for TMA-primed LNC.

The final variable that was investigated was the dosing regimen. LNC were isolated 8 days after the initiation of exposure and a single cell suspension prepared (Vandebriel *et al.*, 2000). This protocol resulted in marked activation of draining lymph

nodes, with cell counts of 12.4 and 11.8×10^6 cells node^{-1} and 16.7 and 18.7×10^6 cells node^{-1} recorded for DNCB- and TMA-activated LNC, respectively, compared with 1.8 and 2.5×10^6 cells node^{-1} observed for LNC isolated from AOO-treated mice. Cells were cultured at 10^6 cells ml^{-1} in the presence of $5 \mu\text{g}$ ml^{-1} of con A or at 10^7 cells ml^{-1} in the presence of medium alone for 24, 72 and 120 h and cytokine content of supernatants determined by cytokine microarray (Fig. 4; two independent experiments). In the absence of con A restimulation, AOO-treated LNC secreted relatively low levels of IFN- γ , IL-4 and IL-10. DNCB- and TMA-activated LNC both secreted detectable, although somewhat variable, levels of IFN- γ . In both experiments, TMA-stimulated LNC expressed considerably higher levels of the type 2 cytokines IL-4 and IL-10 than did DNCB-activated cells. In the presence of con A, the cytokine secretion patterns were considerably less selective. Similar levels of IFN- γ production were recorded for AOO- and allergen-activated LNC, and restimulation with con A increased expression of the type 2 cytokines IL-4 and IL-10 by LNC derived from DNCB-treated mice such that the distinction between DNCB and TMA-activated LNC was less clear. Culture with con A had a similar effect on production of IL-5 and IL-13 by allergen-activated LNC, obscuring the preferential type 2 profile of LNC isolated from TMA-exposed animals (data not shown).

Discussion

Cytokine secretion profiles provoked by DNCB and TMA under various culture conditions have been examined. The use of mitogen, particularly con A, enhanced cytokine expression such that detectable production was recorded even after short-term culture of relatively low concentrations of cells (24 h of culture of cells at 10^6 cells ml^{-1}). However, restimulation with mitogen had a marked effect on the selectivity of the response, with increased cytokine secretion by control vehicle-treated cells. Preferential type 2 cytokine production was largely maintained by TMA- compared with DNCB-activated LNC in the presence of con A and anti-CD3 antibody. However, IFN- γ expression was no longer selective for DNCB-stimulated LNC. The optimal culture conditions for discriminating between the chemical contact and respiratory allergens DNCB and TMA were culture of draining LNC at 10^7 cells ml^{-1} for 120 h in the absence of mitogen. Furthermore, a 13 day exposure period was required for vigorous expression of IFN- γ by DNCB-activated LNC, whereas type 2 cytokine expression by TMA-stimulated LNC was recorded after 8 days. These data confirm earlier reports in which it was shown that con A-activated LNC derived from chronically (13 days) DNCB- and TMA-treated animals expressed similar levels of IFN- γ whereas considerably higher levels of IL-4 were observed for TMA-activated LNC (Vanderbriel *et al.*, 2003). In these experiments, in the absence of mitogen, higher levels of spontaneous production of IFN- γ were detected from DNCB- compared with TMA-stimulated LNC, although these did not reach statistical significance (Vanderbriel *et al.*, 2003). It is of note that the culture period used in the previous studies (Vanderbriel *et al.*, 2003) was 72 h rather than 120 h, a time point which in our experience is suboptimal for spontaneous secretion of this cytokine (Dearman *et al.*, 2002b). The current investigations focused on the reference allergens DNCB and TMA and were not extended to a wider range of allergens. However, the optimal conditions that were identified have been used previously for a variety of respiratory allergens, including platinum salts, various diisocyanates, a range of additional acid anhydrides and cyanuric chloride, all of which provoked a selective

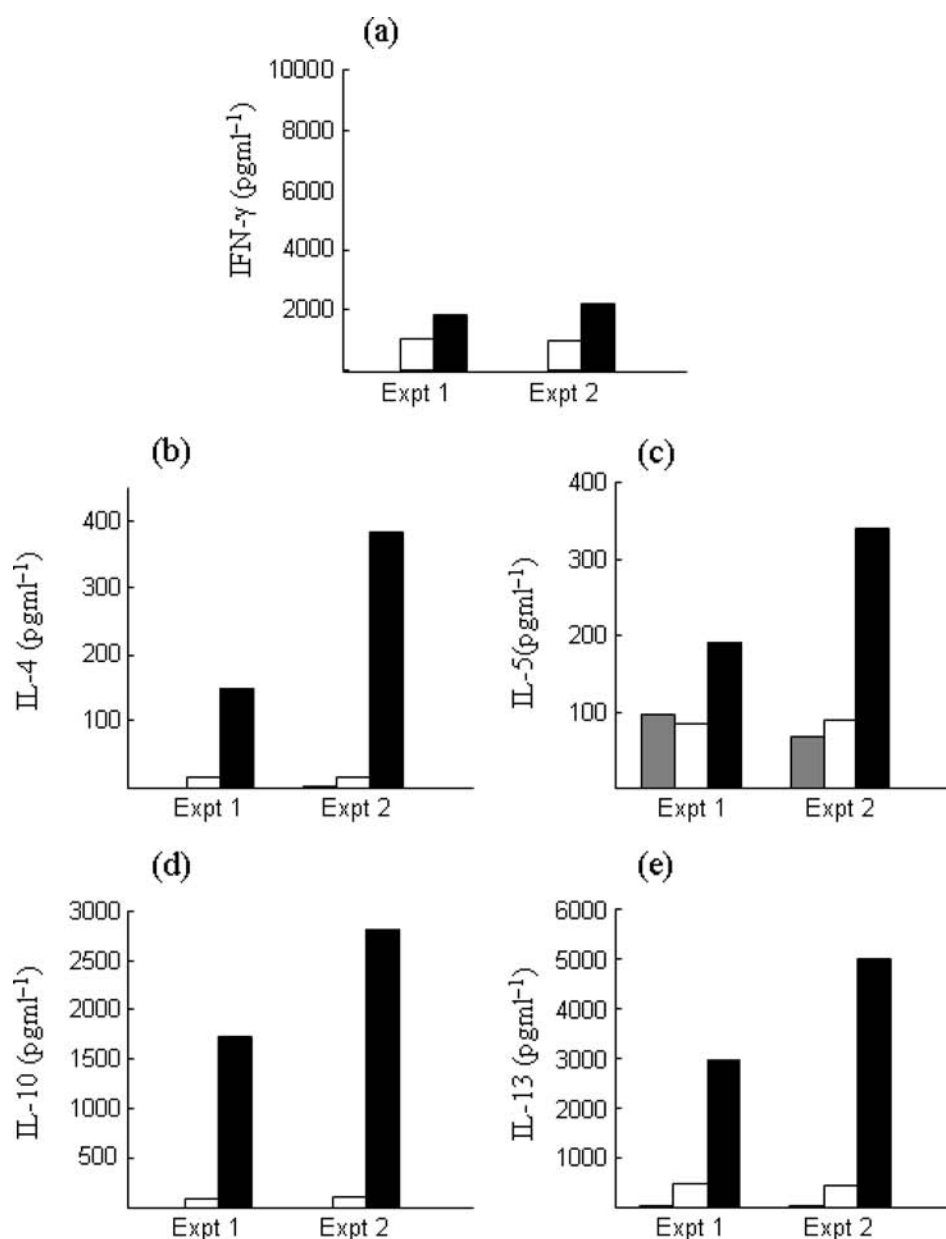


Figure 3. Cytokine expression profiles induced by restimulation with anti-CD3 antibody. BALB/c strain mice were exposed topically to 1% DNCB ($\square$) or to 10% TMA ($\blacksquare$), or to AOO vehicle alone (shaded bar) ($n = 5$ for chemical allergen; $n = 10$ for AOO). Thirteen days after the initiation of exposure, draining lymph nodes were excised, pooled on a treatment group basis and a single cell suspension prepared. Cells were cultured at 10^6 cells ml^{-1} in tissue culture plates pre-coated with $5 \mu\text{g ml}^{-1}$ of anti-CD3. Cells were cultured for 24 h and culture supernatants isolated. Cytokine concentration was determined by cytokine bead microarray (a, IFN- γ ; b, IL-4; c, IL-5; d, IL-10; e, IL-13). Data are shown as pg ml^{-1} for two independent experiments.

type 2 cytokine secretion profile (Dearman and Kimber, 2001; Dearman *et al.*, 2003). Identical treatment with contact allergens, including dinitrofluorobenzene, dinitrothiobenzene, isoeugenol and hexyl cinammic aldehyde, resulted in the converse type 1 cytokine secretion pattern (Dearman and Kimber, 2001; Dearman *et al.*, 2003).

In the current investigations, the cytokine content of supernatants was analysed by microbead array (Luminex) technology. This method allows for the analysis of multiple cytokines in small volumes ($<50 \mu\text{l}$) and is approximately 10-fold more sensitive than enzyme-linked immunosorbent (ELISA) techniques. For example, in previous investigations using ELISA analyses, it proved necessary to restimulate allergen-activated LNC *in vitro* with the

T cell mitogen con A in order to provoke detectable expression of IL-4 (Dearman *et al.*, 2002b). In the current investigations, allergen-induced IL-4 production was observed in the absence of restimulation, with higher levels measured in TMA- compared with DNCB-activated LNC. An additional advantage is that the smaller sample size required for analysis allows for measurement of responses at an individual animal level rather than in samples pooled on an experimental group basis and for a reduction in the number of animals required. In the current experiments, lymph nodes have been pooled for experimental animals because a number of different parameters have been investigated simultaneously; once the protocol has been optimized, sufficient cells should be available for analysis at an individual animal level.

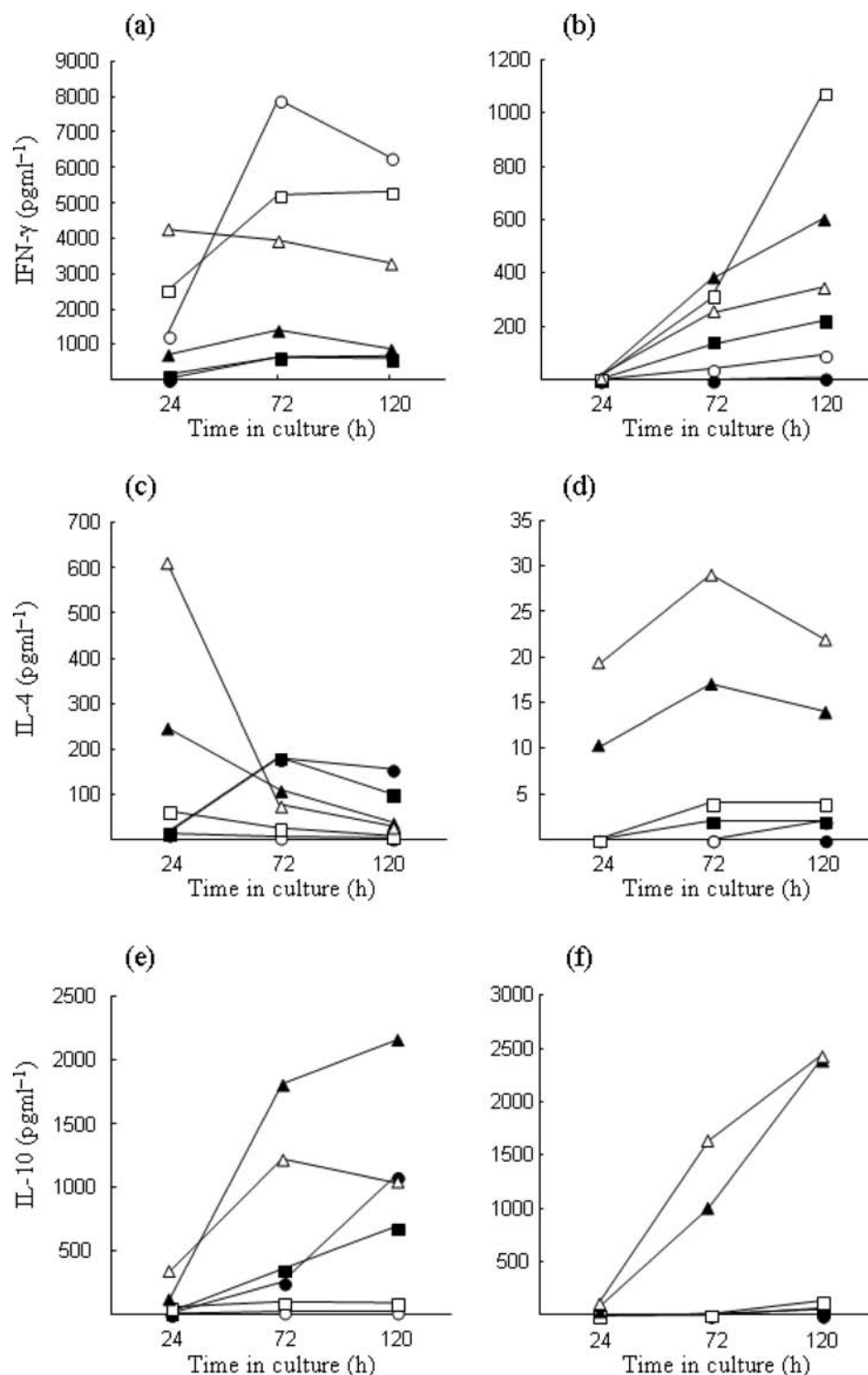


Figure 4. Cytokine expression profiles of allergen-activated lymph node cells: effect of exposure regimen. BALB/c strain mice were exposed topically to 1% DNCB (■) or to 10% TMA (▲), or to AOO vehicle alone (●) ($n = 5$ for chemical allergen; $n = 10$ for AOO). Eight days after the initiation of exposure, draining lymph nodes were excised, pooled on a treatment group basis and a single cell suspension prepared. Cells were cultured at 10^6 cells ml^{-1} in the presence of $5 \mu\text{g ml}^{-1}$ of con A (a, c and e) or at 10^7 cells ml^{-1} in the presence of medium alone (b, d and f). Cells were cultured for 24, 72 and 120 h and culture supernatants isolated. Cytokine concentration was determined by cytokine bead microarray (a and b, IFN- γ ; c and d, IL-4; e and f, IL-10). Data are shown as pg ml^{-1} for two independent experiments (open and closed symbols, respectively).

Immobilized anti-CD3 antibody was relatively ineffective at generating cytokine production by LNC derived from resting (quiescent) lymph nodes. This is consistent with previous observations that naive T cell responses required a strong T cell receptor

signal (high-dose anti-CD3 antibody) and concomitant costimulatory signals (such as anti-CD28 antibody), whereas effector cells responded efficiently to anti-CD3 antibody alone (Dubey *et al.*, 1996). In contrast, culture of naive LNC with the lectin

con A resulted in particularly vigorous production of IFN- γ with relatively low levels of type 2 cytokines. This type 1 cytokine profile for naive LNC has been reported previously at both the message and protein secretion levels (Betts *et al.*, 2004) and presumably reflects polyclonal activation of both CD4⁺ and CD8⁺ T cells (Sander *et al.*, 1991). For allergen-primed cells, stimulation with mitogen resulted in a loss of discrimination between DNCB- and TMA-induced cytokine profiles, particularly for the expression of IFN- γ . These data are consistent with results described for the cytokine secretion profiles of protein-allergen primed spleen cells obtained following restimulation *in vitro* by specific antigen compared with mitogen (Yang and Hayglass, 1993). Splenocytes derived from ovalbumin-sensitized mice expressed selectively high levels of IL-4 when restimulated *in vitro* with protein allergen, but when stimulated with T cell mitogens con A or anti-CD3 antibody, high levels of both IL-4 and IFN- γ were recorded, and similar levels were achieved with mitogen-activated naive cells (Yang and Hayglass, 1993). Although in the absence of mitogen, DNCB- and TMA-activated LNC display preferential type 1 and type 2 cytokine secretion phenotypes, respectively, these are not absolute. Indeed, we have shown previously that exposure to TMA results in increased numbers of CD4⁺ IL-4 secreting cells compared with DNCB; however both treatments result in similar numbers of CD8⁺ IFN- γ secreting cells (Dearman *et al.*, 2005). It is presumably these latter cells which express the high levels of IFN- γ observed following restimulation of TMA-activated LNC with con A (and anti-CD3 antibody).

These data also illustrate the need for an extended (day 13) protocol for the development of polarized cytokine responses, particularly with respect to the production of the type 1 cytokine IFN- γ . This is likely to reflect the fact that polarized forms of T cell develop with time or following repeated exposure from precursor cells, which have the potential to express both type 1 and type 2 cytokines (Grogan *et al.*, 2001). Thus, following acute (3 day) exposure of mice to both DNCB and TMA, vigorous production of IL-4 and IFN- γ by draining LNC is observed (Betts *et al.*, 2002; Moussavi *et al.*, 1998). It is of some interest that 13 days is required for the development of selective type 1 responses to DNCB, whereas within 7 days there is good evidence for preferential type 2 cytokine production by TMA-activated LNC that is not obscured by restimulation with mitogen. It has been demonstrated that differential expression of some marker genes indicative of T helper (Th)1 and Th2 polarization results in within 2 days of culture *in vitro*, with other markers appearing within 7 days (Lund *et al.*, 2003). However, this represents culture conditions that have been designed to be optimal for induction of differentiated cytokine phenotypes in purified naive murine CD4 Th cells in the presence of optimal conditions for culture *in vitro* and may or may not correlate with kinetics of immune responses provoked *in vivo*.

Another important variable in protocols for assessing allergenicity as a function of cytokine secretion profiles is whether cytokines are measured at the level of message or of protein. Changes in cytokine gene expression have been measured using either reverse transcription polymerase chain reaction (RT-PCR) (Dearman *et al.*, 2008; 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; Dearman *et al.*, 2008; Manetz *et al.*, 2001; Plitnik *et al.*, 2002, 2005; Selgrade *et al.*, 2006). Exposure to respiratory allergens including TMA or toluene diisocyanate resulted in increased levels of type 2 mRNA expression compared with treatment with contact allergens such as

DNCB (Dearman *et al.*, 2008; Hayashi *et al.*, 2001; Ryan *et al.*, 1998; Warbrick *et al.*, 1998). However, the type 1 cytokine IFN- γ did not discriminate effectively between contact and respiratory allergens when measured at the level of mRNA by either RT-PCR or RPA (Dearman *et al.*, 2008; Hayashi *et al.*, 2001; Manetz *et al.*, 2001; Plitnik *et al.*, 2002, 2005; Selgrade *et al.*, 2006; Vandebriel *et al.*, 2000; Warbrick *et al.*, 1998). Given the observation that IFN- γ mRNA expression is not increased in DNCB-activated LNC despite robust secretion of this cytokine, the suggestion is that production of this cytokine is controlled mainly at the level of translation of previously transcribed mRNA or of protein secretion.

In our hands, the optimal method of cytokine fingerprinting to permit discrimination between contact and respiratory allergens is a chronic (13 day) exposure regime followed by culture of draining LNC at 10^7 cells mL^{-1} for 120 h in the absence of mitogen for measurement of secreted protein. We would therefore recommend that new materials be tested for respiratory sensitizing potential using this methodology with concurrent TMA- and DNCB-treated control groups. If the cytokine profile of the new material is similar to TMA, it is likely to be a respiratory allergen. A type 1 pattern similar to DNCB would indicate lack of respiratory sensitizing potential.

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