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Immunocytochemical and flow cytofluorimetric detection of intracellular IL-4, IL-5 and IFN- γ : applications using blood- and airway-derived cells

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Abstract

We have compared an immunocytochemical and a flow cytofluorimetric method to detect intracellular IFN- γ , IL-4 and IL-5 in T-cell clones, peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage fluid (BALF) cells. Intracellular bound cytokine-specific antibodies were visualized either with amino-ethyl carbazole (for immunocytochemistry), or with fluorescent antibodies (for flow cytofluorimetry). The staining was inhibited with recombinant cytokines and corresponded qualitatively and quantitatively to cytokine levels in the supernatants of T-helper-0 (Th0), Th1 and Th2 clones. In analysing in vitro stimulated cells, sufficient signal in the fluorimetric assay was only obtained after the addition of monensin to the cultures. We then observed a good correlation between immunocytochemical (with no monensin added) and the flow cytofluorimetric staining for all three cytokines (PBMC, IFN- γ and IL-4, $\rho = 0.9$, no IL-5 detectable; clones, IL-5, $\rho = 0.81$, all three $p < 0.05$). However, compared to flow cytometry, a greater percentage of positively stained cells was frequently observed using immunocytochemistry. In BALF cells, the immunocytochemical method was able to detect significant percentages of positive cells without in vitro stimulation of the cells, in contrast to the flow cytofluorimetric method. In BALF cells from sarcoidosis patients, T-cells were mainly IFN- γ -positive (immunocytochemically assessed), both with (mean $\pm$ SEM, $39.7 \pm 9.8\%$), and without ($3.5 \pm 1.3\%$) in vitro stimulation. In BALF cells from allergic subjects, the immunocytochemical method showed lymphocytes positive for IFN- γ ($40.3 \pm 8.3\%$), IL-4 (19.1 ± 0.49) and IL-5 (6.1 ± 3.1). We conclude that both methods can be used to assess the production of IFN- γ , IL-4 or IL-5 at the single-cell level in T-cell clones, PBMC and cells from the BALF. The high sensitivity and the low number of cells required for the immunocytochemical method indicate that this method can provide detailed information on cytokine production of airway-derived cells in diseases with airway inflammation such as sarcoidosis and asthma.

Keywords: Intracellular cytokine; Immunocytochemistry; Flow cytofluorimetry; Bronchoalveolar lavage; Asthma; Sarcoidosis

Abbreviations: BAL(F), Bronchoalveolar lavage (fluid); IFN, Interferon; IL, Interleukin; PBMC, Peripheral blood mononuclear cells

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1. Introduction

Cytokines are important mediators in physiological and pathological inflammatory responses. Information on cytokine levels and production may, therefore, provide insight into the pathophysiology of diseases. For this purpose, cells and secretory products obtained from the blood or the affected site can be studied. Thus, for pulmonary diseases, such as allergic asthma and sarcoidosis, bronchoalveolar lavage fluid (BALF) may provide important information.

Several methods have been used to study cytokines from cells in the airways. Cytokine proteins secreted in the airways have been measured in BALF (Broide et al., 1992; Walker et al., 1992; Zangrilli et al., 1995). However, this procedure does not reveal the cell type responsible for production. Another approach is to assess the expression of cytokine mRNA in cells from the BALF (Robinson et al., 1992; Huang et al., 1994; Ying et al., 1995). Double-staining techniques can also be used to determine the phenotype of the cells expressing the mRNA. However, translation does not inevitably follow expression of mRNA (Kaldy and Schmitt-Verhulst, 1991; Warren et al., 1991; Lederer et al., 1994; Tang et al., 1994). Cells from BALF can be cultured and *in vitro* cytokine production can then be assessed within a short time, giving general information on the total cell population (Pinkston et al., 1983; Robinson et al., 1985; Walker et al., 1992; Prior et al., 1996). Also, T-cell clones can be generated for analysis at the single-cell level (Garlepp et al., 1992; Hol et al., 1992). The latter method can provide detailed information on the *in vitro* cytokine production pattern of the cultured cells. However, long-term culture may influence the properties of the cells. Thus, cytokine profiles of cell lines or T-cell clones may not be a true reflection of the characteristics of the original cell population.

In assessing cytokine production at the single-cell level in cells from the blood and the airways, we decided to study the application of procedures to detect intracellular cytokines. The presence of cytokine protein in the cell can be detected with a flow cytofluorimetric technique using fluorescent-labelled antibodies (Andersson et al., 1988; Sander et al., 1991; Jung et al., 1993). Information thus obtained is

a fairly accurate representation of the *in vivo* situation as cytokine production profiles can be assessed in freshly obtained cells that have been activated *in vitro* for several hours. In addition, double-staining can serve to identify the type of cell producing the cytokines. Recently, an immunocytochemical method was described for the staining of cytokine proteins in cells on slides for microscopic analysis (Dolhain et al., 1993). This method may be particularly suited to the analysis of cells from BALF since small numbers of cells are needed. Moreover, it has been suggested that this technique is sensitive enough to detect cytokines in cells activated *in vivo*, without any required *in vitro* reactivation (Dolhain et al., 1993).

In the present study, we have compared such an immunocytochemical method with a flow cytofluorimetric procedure to detect intracellular IFN- γ , IL-4 and IL-5 in T-cells. T-cell clones with various cytokine production profiles were used to validate the labelling procedures. Both methods were compared using T-cell clones as well as freshly obtained peripheral blood mononuclear cells (PBMC). We used BALF cells from patients with sarcoidosis to compare both methods since they contain a high percentage of lymphocytes. Various studies have shown an increased production of IFN- γ and not IL-4 and IL-5 in the airways of patients with sarcoidosis (Robinson et al., 1985; Walker et al., 1994). For this reason, we investigated whether staining of intracellular cytokines in these BALF cells also revealed similar patterns. We also explored the application of the immunocytochemical method using cells from the BALF of allergic subjects in order to evaluate its capacity to detect intracellular IL-4 and IL-5 in immunopathological processes skewed towards this cytokine profile.

2. Materials and methods

2.1. Study populations

The following subjects participated in the studies. (1) Six healthy persons who showed normal lung function (with a forced expiratory volume in 1 s (FEV₁) greater than 90% of predicted) and normal bronchial reactivity (the concentration of histamine causing a fall in FEV₁ of more than 20% (PC₂₀) was greater than 8 mg/ml). All subjects were non-

smokers, and they had no history of allergy; their sera showed negative RAST for 5 common aeroallergens. (2) Ten allergic subjects (8 non-smokers) who showed normal lung function ($FEV_1 > 90\%$ of predicted; normal peak flow). The median PC_{20} was 10.8 mg/ml (range 4–16 mg/ml). All allergic subjects had positive results to the skin prick tests and a positive RAST for house dust mite allergens or grass pollen. Three subjects had a history of asthma, four had allergic rhinitis and three were asymptomatic. (3) Six sarcoidosis patients (non-smokers). Sarcoidosis was diagnosed on the basis of an abnormal chest radiograph, a positive 67 gallium scan, and was proven by means of lung biopsy. The sarcoidosis patients had no history of allergy or of asthma. They had negative RAST for common aeroallergens. None of the subjects was on regular medication; in particular, they had not used glucocorticosteroids for at least 3 months prior to the study. None of the subjects had shown signs of airway infections during a period of 6 weeks prior to the study. All gave their written consent for participation. The study was approved by the hospital's medical ethics committee.

2.2. Sample collection

The bronchoalveolar lavage was performed as previously described (Jansen et al., 1984). Briefly, after premedication with atropine and codeine and local lidocaine anaesthesia, a flexible fiberoptic bronchoscope was introduced into the middle lobe of the right lung. Seven aliquots of 20 ml of warmed NaCl (154 mM) were instilled and aspirated immediately. Aliquots 4–7 were pooled and used for the experiments described here. The cells were separated by centrifugation at $500 \times g$ for 10 min at 4°C and washed with phosphate-buffered saline (PBS, 10 mM sodium phosphate, 140 mM NaCl, pH 7.4) containing 0.5% (w/v) bovine serum albumin (Organon Teknika, Boxtel, The Netherlands). Cytospins were prepared and stained by Jenner Giemsa for a differential count of the cells. When necessary, mucolysis was performed using dithiotreitol (0.5 g/l, Sigma, St. Louis, MO) and DNase (Sigma, 16 $\mu\text{g}/\text{ml}$) in a 0.05 M Tris-HCl buffer (Merck, Munich, Germany, pH 7.6).

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of the blood over Ficoll-

Paque (Pharmacia, Uppsala, Sweden). Cells from blood and BALF were immediately processed for the cytokine staining procedures.

$CD4^+$ T-cell clones from blood and BALF were generated using phytohemagglutinin-A (PHA), irradiated peripheral blood mononuclear cells and IL-2 (20 U/ml) (Hol et al., 1992; Krouwels et al., 1996). T-cells had been seeded at 0.3 cell per well, and at 1 cell per well by an automated cell deposition device. Allergen-specific T-cell clones were obtained from T-cell lines cultured in the presence of allergen and antigen presenting cells. These cultures were performed in the presence of house dust mite major allergen (*Der p 2*) (Van Neerven et al., 1993). On the basis of previously determined cytokine profiles, we selected T-helper-1 clones (Th1) producing mainly IFN- γ , Th2 clones producing mainly IL-4 and IL-5, and Th0 clones producing all three cytokines.

Cells were stored at 2×10^6 cells/ml in Iscove's modified Dulbecco's medium (Gibco, Paisley, UK) supplemented with 10% (v/v) heat-inactivated pooled human serum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands), 2×10^{-5} M β -mercaptoethanol (Merck), 2 mM Na-pyruvate (Merck), penicillin (100 U/ml, Gist-Brocades, Delft, The Netherlands) and streptomycin (100 $\mu\text{g}/\text{ml}$, Gibco). The kinetics of cytokine production were investigated by analysing unstimulated cells or cells stimulated with phorbol myristate acetate (PMA, 1 ng/ml, Sigma) plus ionomycin (500 ng/ml, Sigma) $\pm$ monensin (Sigma) at the indicated concentrations, and incubated at 37°C in humidified air containing 5% CO_2 for 2, 4, 6, 16 or 24 h. At each time point, supernatants were collected for subsequent measurements of the concentrations of IL-4, IL-5 and IFN- γ by ELISA. The stimulated cells were washed and resuspended at $0.5\text{--}2 \times 10^6$ cells/ml before staining for analysis by immunocytochemistry or flow cytometry. When the optimum period of stimulation required for maximal intracellular cytokine production had been determined, cells were only cultured for this length of time in subsequent experiments.

For the ELISA of IFN- γ we used monoclonal antibodies (MAbs) anti-IFN- γ MD2 and biotinylated MD1 (Van der Meide et al., 1985). IL-5 was mea-

sured using MAb TRFK-5 anti-IL-5 (coating concentration 1 µg/ml) and biotinylated JES1-5A10 anti-IL-5 (1 µg/ml) (Pharmingen, San Diego, CA) following the manufacturers instructions. After incubation with the detecting antibody, plates were incubated with streptavidin–poly-HRP (CLB, 1/10000 v/v) in PBS containing 2% (v/v) cow's milk, and subsequently developed with tetramethylbenzidine (Sigma) and H₂O₂. IL-4 was measured by ELISA using a commercial kit (CLB).

2.3. Reagents and antibodies for intracellular labelling

Blocking of non-specific binding was performed with normal goat serum (Bio Trading, Meidrecht, The Netherlands). Permeabilization buffer was PBS containing 0.1% (v/v) saponin (Sigma). Fixation was performed with paraformaldehyde (PFA, Merck) in 0.1 M sodium phosphate buffer.

The following MAbs were used for intracellular cytokine staining. Anti-IFN-γ: MD2 (Van der Meide et al., 1985). A cocktail of anti-IL-4 MAbs: 8F-12, 4D-9 and 1G-1 (all mouse IgG1 (Andersson et al., 1990)). Anti-IL-5: either 39D10 (Genzyme, Emeryville, CA) or biotin-labelled JES1-5A10. The final concentrations of the antibodies during the labelling procedures were 10 µg/ml. Biotin-labelled goat–anti-mouse IgG1: E433, and HRP-labelled streptavidin: P497 were obtained from DAKO (Santa Barbara, CA). Irrelevant monoclonal antibodies: X40 and X39 and phycoerythrin (PE)-labelled streptavidin were from Becton-Dickinson (Mountain View, CA). The aminoethyl-carbazole solution (AEC) contained AEC (Sigma, 10 mg/ml), *N,N*-dimethylformamide (1 mg/ml), 0.1% (w/v) saponin and 0.05% (v/v) H₂O₂ in 50 mM sodium acetate (pH 4.9). This solution was freshly prepared and filtered before use. Glycergel was obtained from DAKO. Recombinant human (rh) IL-4, IL-5 and IFN-γ were from Genzyme.

2.4. Staining procedure for light microscopy

We used the method of Dolhain et al. (1993) with minor adaptations. The cells were collected, washed with ice-cold PBS and resuspended at $0.5\text{--}2 \times 10^6$ cells/ml. Subsequently, they were mounted on num-

bered, positively charged adhesion slides (Biorad, Munich, Germany) by adding 10 µl of the cell suspension per reaction field. All further steps were performed at room temperature, except when stated otherwise. The cells were allowed to adhere in a humidified atmosphere for 10 min. After fixation with 4% (w/v) PFA (30 min), the cells were incubated for 10 min with 10% (v/v) normal goat serum in PBS to prevent non-specific binding. The cells were then made permeable by carefully rinsing the slides 3 times with 5 ml PBS containing 0.1% (w/v) saponin. This buffer was used in all further steps. 10 µl of the antibody suspension, containing either anti-IFN-γ, anti-IL-4, anti-IL-5 or irrelevant isotype matched antibodies were added to each reaction field and incubated at 37°C for 30 min. After a wash, the cells were incubated with biotin-labelled goat-anti-mouse (1/200 diluted) for 30 min. The cells were washed and incubated with 20 µl of streptavidin–HRP (1/400) for 30 min. Next, the cells were washed and treated with the AEC solution until staining was visible with the light microscope. The cells were then washed, embedded in glycergel and scored with a light microscope within 1 week. The low endogenous peroxidase activity in monocytes and macrophages was easily discernible from the specific signal generated by the anti-cytokine antibodies. All slides were analysed and scored independently by two investigators. After scoring by both investigators, the origin of the slides was identified. The staining remained stable for at least 1 year.

2.5. Staining procedure for flow cytometry

Cells were kept in tubes on melting ice at all times. The cells were collected, washed and resuspended to $2\text{--}5 \times 10^5$ cells in 150 µl PBS (Jung et al., 1993). Then 50 µl of PFA (4%, w/v) were added to the cell pellet, and incubated for 10 min. Blocking, permeabilization, washing, and incubation with the anti-cytokine MAbs were performed in tubes at a volume of 100 µl and temperature of 0°C. For blocking experiments, the cells were incubated for 10 min with 10% (v/v) normal goat serum in PBS. Next, the cells were pelleted ($400 \times g$ for 5 min) and the medium decanted; the cells were resuspended in the permeabilization buffer (0.1% (w/v) saponin in PBS) at 0°C, and immediately centrifuged for 5 min;

this procedure was repeated twice. Monoclonal anti-cytokine antibodies were incubated with the cells for 30 min at 0°C. Subsequently, the cells were incubated for 30 min with biotin-labelled goat-anti-mouse (1/200) and, after washing, with streptavidin-PE (1/50) for 30 min. Between each step, cells were washed and collected by centrifugation. Fluorescence intensity was measured with a flow cytometer (FACScan, Becton-Dickinson). Lymphocytes were gated on the basis of forward and side light scatter. In all experiments, parallel incubations were performed with irrelevant antibodies matched for the isotypes of the anti-cytokine antibodies. The cut-off level for definition of positive cells was thus set so that less than 1% of irrelevant antibody stained cells were positive.

To evaluate the specificity of the immunocytochemical staining, blocking experiments were performed. To this end, recombinant cytokines were added at increasing concentrations, starting from 0.05 ng/ml, to the solutions containing the corresponding antibodies. These solutions were centrifuged to remove complexes and subsequently added to the cells. There was gradual decrease in positive staining with the following maximal inhibition: 90% inhibition at 10 ng rhIFN- γ /ml; IL-4, 99% at 0.5 ng/ml; IL-5, 38% at 300 ng/ml. In the cytofluorimetric method blocking experiments were performed with rhIFN- γ only. Maximal inhibition was 82% at 10 ng rhIFN- γ per ml.

2.6. Statistical analysis

The Spearman rank test was used to calculate correlations between two variables. The Mann-Whitney *U*-test was used to evaluate differences between unpaired groups. Probability values less than 0.05 were considered significant. In comparing results obtained by two observers, each individual's scores were grouped in 10 categories: 0–1.0% positive cells, 1.1–5.0%, 5.1–10.0%, 10.1–20.0%, 20.1–30.0%, 30.1–40.0%, 40.1–50.0%, 50.1–60.0%, 60.1–80.0% and 80.1–100% positive cells. Using the distribution of each observer's score, the weighted κ was calculated. Conformity between both observers was considered high if the weighted κ was greater than 0.81 (Altman, 1995).

3. Results

3.1. Intracellular staining of cytokines in T-cell clones: immunocytochemistry

3.1.1. Characteristics of intracellular staining of cytokines

Unstimulated clonal T-cells did not show staining for either IL-4, IL-5, or IFN- γ (illustrated in Fig. 1a for IFN- γ). After stimulation with PMA and ionomycin (PMA/IONO), cells stained positive for each of the three cytokines. A bright red spot observed adjacent to the nucleus (shown in Fig. 1b for IFN- γ in a Th1 clone) is typical for proteins accumulating in the ER-Golgi (Sander et al., 1991). Similar staining was also found after labelling for IL-4 and IL-5 (Fig. 1d and f, respectively).

The scoring of positive cells was highly reproducible. The scores of two observers were compared by calculating the weighted κ in a panel of 12 T-cell clones with a large variation in the percentage of cytokine-positive cells. It was higher than 0.9 for all cytokines, indicating a high level of reproducibility.

3.1.2. Time course of staining of intracellular cytokine proteins

Fig. 2 shows the time course of the appearance of intracellular cytokines. The cells stained positive for cytokines from 2 h after stimulation onwards. The highest percentage of positively stained cells was found at 4 h (IL-4 and IL-5) or 6 h (IFN- γ). Staining for IL-4 and IL-5 declined strongly after 4 h, whereas the percentage IFN- γ -positive cells was relatively stable for at least 20 h. After 24 h, 2–6% IL-5-positive and no IL-4-positive cells were found. In further experiments, the intracellular staining for cytokine proteins was evaluated 4 h after stimulation. Staining for IFN- γ on clonal T-cells that had been stimulated and adhered on 1 day and stained on different days was reproducible: IFN- γ positive cells, $55 \pm 9\%$ (mean $\pm$ SD, $n = 5$). When we stimulated and stained T-cell clones on different days the results were as follows; positive cells for IFN- γ : clone 1, $47 \pm 9\%$ (mean $\pm$ SD, $n = 7$); IL-4: clone 2, $26 \pm 9\%$ ($n = 6$).

Cytokine protein released in cell supernatants was also detected from 2 h after stimulation onwards; maximal concentrations were found after 16 or 24 h

Fig. 2. Time course of appearance of intracellular cytokines in T-cell clones. Four different Th0 clones were activated with PMA/IONO. Cells and supernatants were harvested at several time points. The filled dots indicate the mean and SD ($n=4$) of the positively stained cells; the bars indicate the mean and SD ($n=4$) of the cytokine concentrations in the supernatants.

Table 1
Intracellular and secreted cytokines in T-cell clones

	% Positive cells			Supernatant (ng/ml)		
	IFN- γ	IL-4	IL-5	IFN- γ	IL-4	IL-5
Th1 clones						
1	90%	0%	0%	11.0	0.008	0.1
2	83%	0%	0%	9.1	0.02	0.1
Th2 clones						
4G10	< 1%	53%	27%	< 0.03	2.2	1.4
3C5	10%	100%	100%	0.1	4.2	7.6
3F7	22%	88%	49%	0.64	3.6	1.8
Th0 clones						
3B1	74%	37%	24%	8.5	1.2	6.4
9C7	59%	50%	20%	2.3	2.5	1.9
27D9	53%	25%	7%	3.4	0.9	1.7
4	53%	38%	15%	2.0	3.1	0.9
5	21%	74%	53%	1.0	4.0	3.0

Cytokine profiles of T-cell clones measured as intracellular and secreted cytokines. Cells were stimulated with PMA/IONO for 4 h (for intracellular staining) or 24 h (for measurement in the supernatants).

(Fig. 2). In a panel of 12 Th0 clones, producing all three cytokines, the percentage of positive cells found at 4 h was closely related to the cytokine concentration in the supernatants collected 24 h after stimulation (Spearman's correlation ρ : IFN- γ , 0.93; IL-4, 0.93; IL-5, 0.94; for each cytokine, $p < 0.001$; illustrated in Table 1 for 5 Th0 clones).

3.1.3. Th1 / Th2 cytokine profiles are reflected in the intracellular staining for IFN- γ , IL-4 and IL-5

T-cell clones with a Th1- and Th2-type cytokine profile (based on the cytokine release in the supernatants) were used to study whether such a distinction could also be made on the basis of staining of intracellular cytokines. In a stimulated Th1 clone, we found staining for IFN- γ only, and not for IL-4 or IL-5 (Fig. 1 and Table 1). In Th2 clones, some IFN- γ was found, both intracellular and in the super-

Fig. 1. Staining for intracellular cytokines in T-cell clones (a–f). Magnification $\times 200$. Note that the cells adherent to the slide retained their three-dimensional structure. Therefore, a photograph taken at one level reveals only part of the positive cells. (a) No staining in unstimulated cells, labelled with anti-IFN- γ . (b) Th1 cells, stimulated for 4 h with PMA/IONO. In 90% of the cells a red spot is found next to the nucleus representing the accumulation of IFN- γ in the Golgi-organelle. (c) Th1 cells activated and stained for IL-4; no positive cells. A similar result was observed for IL-5 staining. (d,e,f) Th2 cells labelled with anti-IL-4 (100% positive cells), IFN- γ (10% positive cells) and anti-IL-5 (100% positive cells), respectively. (g,h) Staining for intracellular IFN- γ in BALF cells from a sarcoidosis patient. The cells in BALF of this patient contained 34% lymphocytes. The percentage IFN- γ positive cells without stimulation was 2% (g) and at 4 h after stimulation was 68% (h); 1, negatively staining lymphocyte; 2, alveolar macrophage; 3, positively staining lymphocyte.

Table 2
Immunocytochemical and flow cytofluorimetric staining in T-lymphocyte clones

T-cell clone	Flow cytofluorimetric staining (% positive cells)						Immunocytochemical staining (% positive cells)		
	Without monensin			With monensin			Without monensin		
	IFN- γ	IL-4	IL-5	IFN- γ	IL-4	IL-5	IFN- γ	IL-4	IL-5
1	16	7.8	1	42	21	1	90	4	1
2	3.1	0.3	1.7	44	0.8	2.7	70	5	1
3	9	6	2.7	15	6.2	3	73	6	1
4	34	6.1	2.7	81	9.5	5.5	74	25	1.5
5	10	12	2	26	16	5	74	35	11
6	9.6	12	1.7	59	21	5.1	60	59	7
7	1.5	10	29	12	25	25	1	69	49

Cytofluorimetric staining in the absence and presence of 2.5 μ M monensin, and immunocytochemical staining in the absence of monensin. The cells were stimulated with PMA/IONO for 4 h with or without the presence of monensin. Cells were collected and handled as described in Section 2: Materials and methods.

natants. However, the intracellular staining for IL-4 and IL-5 and their release was predominant (Fig. 1 and Table 1).

3.2. Intracellular staining of cytokines in T-cell clones: flow cytofluorimetry

As with the immunocytochemical method for T-cell clones, we detected cytokine-positive cells only after stimulation of the cells. The time course of appearance of intracellular cytokines in the flow cytometric analysis was similar to that shown in Fig. 2 for the immunocytochemical procedure. The percentages of positive cells were for Th0 cells ($n = 7$): IFN- γ , median 9.6% (interquartile range 2–19%); IL-4, 8% (4–11%) and IL-5, 2% (1.8–3%). In parallel experiments, monensin, which interrupts subcellular transport and secretion of proteins was added at the start of cultures (Jung et al., 1993). Monensin at a concentration of 0.5–5 μ M enhanced the percentage of positive cells: IFN- γ , 41% (13–60%); IL-4, 18% (4–22%); IL-5, 5% (3–6%). At higher concentrations of monensin, the viability of the cells decreased as judged from light scatter and staining with propidium iodide.

The fluorimetric and cytochemical methods were compared for 7 clones (Table 2). The percentages of cells positive for IFN- γ and IL-4 in the first method correlated well with those in the latter when stimulated without the presence of monensin ($\rho = 0.83$

and 0.68, respectively, $p < 0.05$ and 0.1, respectively). Staining for IL-5 was very low in most clones. For all three cytokines, however, the percentage of positive cells was much higher in the immunocytochemical method than in the immunofluorimetric method (Table 2). When the cells were stimulated in the presence of monensin, the staining in the immunocytochemical method became very intense. This made it impossible to discern cell morphology. We decided, therefore, not to evaluate monensin-treated cells with the immunocytochemical method. The application of monensin in the fluorimetric method appeared to be very useful as it resulted in a considerable increase in the percentage of positive cells in most experiments. However, the percentage of positive cells was still lower than that observed in the immunocytochemical method for most preparations (Table 2). Under those conditions, a significant correlation between both methods was found for IL-5 ($\rho = 0.81$, $p < 0.05$).

3.3. Intracellular staining of cytokines in PBMC

We measured the labelling of intracellular cytokines in stimulated PBMC from 5 healthy subjects using the adhesion slides. Monocytes were discriminated from lymphocytes on the basis of their morphology and did not show significant staining. There was a high degree of correlation between the scores of two observers; the weighted κ was > 0.89 for all

Table 3
Immunocytochemical and flow cytofluorimetric staining in PBMC

Subject	Flow cytofluorimetric staining (% positive cells)						Immunocytochemical staining (% positive cells)		
	Without monensin			With monensin			Without monensin		
	IFN- γ	IL-4	IL-5	IFN- γ	IL-4	IL-5	IFN- γ	IL-4	IL-5
1	7.6	0.7	1	5	2	1.4	17	7	0.5
2	8.4	3.1	0.8	12	8.2	2.1	21	34	1.6
3	24	0.9	2.7	49	4.2	0.6	47	12	1
4	9.6	1.7	1.7	14	6.1	2.7	38	20	1.1
5	8	1.5	1.7	25	5.6	5.5	33	25	7

Cytokine profiles of PBMC from healthy subjects were measured by flow cytofluorimetric and by immunocytochemical staining. PBMC were stimulated with PMA/IONO for 4 h with or without 2.5 μ M monensin. Cells were collected and handled as described in Section 2: Materials and methods.

three cytokines. PBMC from one donor that had been stored frozen were stimulated and stained on different days with the following results: IFN- γ , $17 \pm 3.4\%$; IL-4, $3.8 \pm 2.2\%$ positive cells (mean $\pm$ SD, $n = 4$). The results by immunocytochemistry were compared with those obtained by flow cytometry (Table 3). Again, the highest percentage of IFN- γ and IL-4-positive lymphocytes were found with the first method. Both with and without the presence of monensin during culture, a correlation was found between the percentage of cytokine-positive cells using either method (IFN- γ and IL-4, $\rho = 0.90$; $p < 0.05$).

3.4. Intracellular staining of cytokines in cells from BALF

3.4.1. Sarcoidosis

The BALF cells from patients with sarcoidosis contained a high percentage of lymphocytes (median 32.2%, range 7.0–47.2%). The lymphocytes were readily distinguished from the macrophages on the adhesion slides. A representative result for IFN- γ staining is shown in Fig. 1g,h. The scores of two observers for the staining of intracellular cytokine proteins showed high correlation for all cytokines (weighted $\kappa > 0.88$).

In 3 patients, sufficient cells were available to compare the results obtained by immunocytochemistry with those from flow cytofluorimetry. In the flow cytofluorimetric method, no staining was found in unstimulated cells. Four hours after stimulation with PMA/IONO, the percentage of IFN- γ -positive

cells was 2–3%, and no IL-4- or IL-5-positive cells were detected. With the immunocytochemical method, the percentages of IFN- γ -positive cells found in unstimulated cells were 1, 2 and 5.7%, respectively. Fewer than 1% of the cells were positive for either IL-4 or IL-5. After stimulation, a high percentage of cells stained positive for both cytokines. The following percentages of cells were positive by the

Fig. 3. The percentages of cytokine-positive cells in freshly isolated cells from the BALF as determined by the immunocytochemical method. The cells were analysed without stimulation in vitro (A), and 4 h after stimulation with PMA/IONO (B). Note the different scale for IL-5.

immunocytochemical technique: 68, 55 and 17% for IFN- γ ; 4, 1 and 3% for IL-4, and 0.8, 1 and 0.5% for IL-5, respectively. For flow cytometric analysis, it was necessary to add monensin to the cultures to permit detection of high percentages of positive cells. The figures were as follows: 17, 21 and 29% for IFN- γ ; 15, 1.9 and 2.5% for IL-4; and 23, 0.7 and 1.4% for IL-5. In this small number of experiments, no significant differences between the flow cytometric and immunocytochemical methods were found.

Using the immunocytochemical method, we observed positive staining for IFN- γ in unstimulated BALF cells in 5 of the 6 patients analyzed (range 2–9%, Fig. 3A). Stimulation with PMA/IONO increased the staining for all cytokines in all patients. Strikingly, a high percentage of cells positive for IFN- γ was found, with large inter-individual variations (mean 39.7%, range 9–68%, Fig. 3B). For IL-4 and IL-5, the scores after stimulation were: 6.7% (1–27%) and 1.2% (0–5%), respectively (Fig. 3B).

3.4.2. Allergic subjects

Using the immunocytochemical method, we studied the occurrence of intracellular cytokines in cells from the BALF of 10 allergic subjects. The percentages of lymphocytes in the BALF were: median 2.7%, range 0.2–12.9%. For one subject, the differentiation and the scoring of the cells from the BALF was impossible due to the presence of relatively small macrophages. In two patients, the low numbers of lymphocytes did not permit staining for all three cytokines. The scores of two observers were closely correlated; the weighted κ was > 0.86 for all cytokines.

Without *in vitro* stimulation, cells stained positive for IFN- γ in 4 out of 8 patients, for IL-4 in 2 of the 7, and for IL-5, in 2 out of 7 patients (Fig. 3A). After *in vitro* stimulation, IFN- γ and IL-4 positive cells were found in all patients (ranges: IFN- γ , 11.5–70.9% and IL-4, 2.8–38.5%) (Fig. 3B). Positive staining for IL-5 was much less than for IFN- γ or IL-4 (range 0–27%). IL-5-positive cells were found in 7 of the 8 patients. All of the cells staining positive for either of the cytokines had the morphology of lymphocytes.

The BALF cells from allergic subjects showed a significantly higher percentage of IL-4-positive cells than did similar preparations from patients with sar-

coidosis (Mann-Whitney *U*-test (MWU), $p < 0.05$). The percentage of IL-5-positive cells also tended to be higher, though this difference was not statistically significant (MWU, $p = 0.07$).

4. Discussion

For patients with immunopathological diseases of the airways, the study of cytokine production by cells sampled from the affected site can provide useful information on the pathophysiology of the disease. The analysis of intracellular cytokine proteins may be particularly useful, but is hampered by low numbers of cells in BALF and the suboptimal activation of cells *in vivo*. In this study, we have compared two methods for the detection of intracellular cytokines in T-cells. We were particularly interested in determining the capacity of either method to detect intracellular cytokines when: (1) low numbers of cells are present; and (2) cells have been activated only *in vivo*. We used an immunoperoxidase technique derived from a previously described method (Dolhain et al., 1993) and a flow cytofluorimetric technique (Jung et al., 1993). The specificity of the staining was confirmed with added recombinant cytokines (although staining for IL-5 was not completely inhibited, probably because higher concentrations of added IL-5 are needed (Eliason et al., 1995)). We showed that the percentage score of cytokine-positive cells was both qualitatively and quantitatively related to the profile and amount of secreted cytokines in Th1-, Th2- and Th0-like T-cell clones.

When we used the cytofluorimetric technique to detect intracellular IFN- γ , IL-4 and IL-5 in stimulated PBMC, we found percentages of positively staining cells similar to those reported in earlier studies (Andersson et al., 1988; Labalette-Houache et al., 1991; Sander et al., 1991; Jung et al., 1993; Schmitz et al., 1993). The results of the immunocytochemical method were correlated with those of the flow cytometric method in T-cell clones and in PBMC, though not under all conditions. With the immunocytochemical technique, however, higher percentages of cells were found to be positive for IFN- γ and IL-4. Sander et al. (1991) have reported immunofluorescence microscopy to be a more sensi-

tive detector of intracellular cytokines than flow cytofluorimetry. In addition, Dolhain et al. (1993) obtained a slightly higher percentage of positively staining cells with the immunoperoxidase method than with the immunofluorescence method. This difference probably reflects the fact that a weak signal from a cytokine present in the Golgi organelle is readily detectable by microscopy due to its characteristic morphology. The identification of such a small spot permits the detection of positive cells producing only a small amount of cytokines. This is particularly relevant for the analysis of cells stimulated *in vivo*. In the flow cytometer, the integration of the fluorescent signal over the whole cell decreases the sensitivity.

In addition to the differences in sensitivity, the following aspects should also be considered in comparisons between the immunocytochemical and the immunocytofluorimetric procedures: (1) in the immunocytochemical procedure the numbers of cells required (5 to 10×10^3 cells) are 5–10 times lower than those needed for cytometric analysis; (2) the immunocytochemical staining technique is simple, and the slides can be examined using an ordinary light microscope; (3) the slides can be stored without loss of quality; (4) visual inspection provides a high certainty about the nature of the positive signal; (5) double-staining for subpopulations of cells is easily performed in the flow cytofluorimetric technique, but, in our hands, it was not readily applicable to the immunocytochemical procedure; (6) cells labelled either for surface markers or for intracellular cytokines, may be easily sorted by flow cytometry; (7) inhibition of cytokine secretion by the addition of monensin during *in vitro* stimulation is not required in the immunocytochemical technique. We noticed that when applied to cultures evaluated by the immunocytochemical procedure, staining was so intense that it was no longer possible to determine the cell morphology. This was already observed using as little as $0.1 \mu\text{M}$ monensin. In other studies, Brefeldin A has been used to prevent cytokine secretion by the cells (Openshaw et al., 1995). This agent is less toxic for the cells and its presence during the last 2 h of culture may be sufficient to obtain significant signals from intracellular cytokine proteins.

The cells obtained from the BALF of sarcoidosis patients contained high percentages of lymphocytes.

These cells are known to show signs of recent activation (Costabel et al., 1985; Du Bois et al., 1992; Hol et al., 1993) and spontaneously release IFN- γ when cultured *in vitro* (Robinson et al., 1985). In the BALF of patients with sarcoidosis, elevated levels of IFN- γ were found, whereas the levels of IL-4 and IL-5 were not elevated (Walker et al., 1994). Our finding that unstimulated BAL lymphocytes from most patients with sarcoidosis were positive for IFN- γ and not for IL-4 or IL-5 agrees with those studies. The high IFN- γ potential of these cells was confirmed in this study by the high percentage of IFN- γ -positive cells after *in vitro* stimulation of the BALF cells, whereas staining for IL-4 or IL-5 hardly increased.

IgE-mediated allergy is associated with the production of IL-4 and IL-5 (Romagnani, 1994) and for this reason we studied BALF cells from allergic subjects. It has been reported that the airways of such patients show mucosal eosinophilia, activation of eosinophils and mast cells, and abnormal collagen deposition (Djukanovic et al., 1992). These findings are similar to the characteristic pathophysiological changes observed in patients with allergic asthma, though less marked (Djukanovic et al., 1992). The IL-4 concentration in BALF from allergic asthmatic patients was similar to those from non-asthmatic allergic subjects, and higher than in healthy controls (Zangrilli et al., 1995). We found that in allergic subjects, a high percentage of BALF cells were positive for IL-4 and IL-5 after *in vitro* stimulation of the cells. BALF cells from allergic subjects contained a higher percentage of cells positive for IL-4 and IL-5 than did BALF cells from sarcoidosis patients.

In our hands, both the immunocytochemical and the cytofluorimetric analysis of intracellular cytokines were appropriate for the study of cytokine production by T-cells. In heterogeneous cell populations, where large numbers of T-cells are available, the cytometric technique is preferable, since it makes it possible to focus on subsets of lymphocytes using double or triple staining. In BALF cells, the immunocytochemical method is particularly promising as it can detect intracellular cytokines without additional *in vitro* restimulation. The findings from our studies of patients, though still limited, indicate that BAL cells from sarcoidosis patients and allergic

subjects have a high potential for cytokine production. The application of these observations to larger groups of patients and healthy subjects may provide new insights into the role of cytokines in inflammatory processes in the lung.

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References

- Altman, D.G., 1995. Some common problems in medical research. In: Altman, D.G. (Ed.), *Practical statistics for medical research*. Chapman and Hall, London, UK, pp. 403.
- Andersson, U., Hallden, G., Persson, U., Hed, J., Möller, G., DeLey, M., 1988. Enumeration of IFN- γ -producing cells by flow cytometry. *J. Immunol. Methods* 112, 139.
- Andersson, U., Andersson, J., Lindfors, A., Wagner, K., Möller, G., Heusser, C.H., 1990. Simultaneous production of interleukin 2, interleukin 4 and interferon- γ by activated human blood lymphocytes. *Eur. J. Immunol.* 20, 1591.
- Broide, D.H., Lotz, M., Cuomo, A.J., Coburn, D.A., Federman, E.C., Wasserman, S.I., 1992. Cytokines in symptomatic asthma airways. *J. Allergy Clin. Immunol.* 89, 958.
- Costabel, U., Bross, K.J., Ruhle, K.H., Löhr, G.W., Matthys, H., 1985. Ia-like antigens on T-cells and their subpopulations in pulmonary sarcoidosis and in hypersensitivity pneumonitis. *Am. Rev. Respir. Dis.* 131, 337.
- Djukanovic, R., Lai, C.K.W., Wilson, J.W., Britten, K.M., Wilson, S.J., Roche, W.R., Howarth, P.H., Holgate, S.T., 1992. Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic nonasthmatics and healthy controls. *Eur. Respir. J.* 5, 538.
- Dolhain, R.J.E.M., Andersson, U., ter Haar, N.T., Brinkman, B.M.N., Verweij, C.L., Daha, M.R., Breedveld, F.C., Miltenburg, A.M.M., 1993. Detection of intracellular interferon- γ by light microscopy using an immunoperoxidase technique: correlation with the corresponding mRNA and protein product. *J. Leukocyte Biol.* 54, 545.
- Du Bois, R.M., Kirby, M., Balbi, B., Saltini, C., Crystal, R.G., 1992. T-lymphocytes that accumulate in the lung in sarcoidosis have evidence of recent stimulation of the T-cell antigen receptor. *Am. Rev. Respir. Dis.* 145, 1205.
- Elson, L.H., Nutman, T.B., Metcalfe, D.D., Prussin, C., 1995. Flow cytometric analysis for cytokine production identifies Thelper1, Thelper2, and Thelper0 cells within the human CD4+CD27- lymphocyte subpopulation. *J. Immunol.* 154, 4294.
- Garlepp, M.J., Rose, A.H., Bowman, R.V., Mavaddat, N., Dench, J., Holt, B.J., Baron-Hay, M., Holt, P.G., Robinson, B.W.S., 1992. A clonal analysis of lung T cells derived by bronchoalveolar lavage of healthy individuals. *Immunology* 77, 31.
- Hol, B.E.A., Hintzen, R.Q., Van Lier, R.A.W., Alberts, C., Out, T.A., Jansen, H.M., 1993. Soluble and cellular markers of T-cell activation in patients with pulmonary sarcoidosis. *Am. Rev. Respir. Dis.* 148, 643.
- Hol, B.E.A., Krouwels, F.H., Bruinier, B., Reijneke, R.M.R., Mengelers, H.J.J., Koederman, L., Jansen, H.M., Out, T.A., 1992. Cloning of T-lymphocytes from broncho-alveolar lavage fluid. *Am. J. Respir. Cell Mol. Biol.* 7, 523.
- Huang, S.-K., Krishnaswamy, G., Su, S.-N., Xiao, H.-Q., Liu, M.C., 1994. Qualitative and quantitative analysis of cytokine transcripts in the bronchoalveolar lavage cells of patients with asthma. *Ann. New York Acad. Sci.* 725, 110.
- Jansen, H.M., Schutte, A.J.H., Elema, J.D., Van der Giessen, M., Peset Reig, R., Leeuwen, M.A.V., Sluiter, H.J., The, T.H., 1984. Local immune complexes and inflammatory response in patients with chronic interstitial pulmonary disorders associated with collagen vascular diseases. *Clin. Exp. Immunol.* 56, 311.
- Jung, T., Schauer, U., Heusser, C., Neumann, C., Rieger, C., 1993. Detection of intracellular cytokines by flow cytometry. *J. Immunol. Methods* 159, 197.
- Kaldy, P., Schmitt-Verhulst, A.-M., 1991. Cyclic AMP synergy with Ca^{2+} for production of IFN- γ by a cytolytic T cell clone is post-transcriptional. *J. Immunol.* 146, 2382.
- Krouwels, F.H., Hol, B.E.A., Bruinier, B., Lutter, R., Jansen, H.M., Out, T.A., 1996. Cytokine production by T-cell clones from bronchoalveolar lavage fluid of patients with asthma and healthy controls. *Eur. Respir. J.*, in press.
- Labalette-Houache, M., Torpier, G., Capron, A., Dessaint, J.P., 1991. Improved permeabilization procedure for flow cytometric detection of internal antigens; analysis of IL-2 production. *J. Immunol. Methods* 138, 143.
- Lederer, J.A., Liou, J.S., Todd, M.D., Glimcher, L.H., Lichtman, A.H., 1994. Regulation of cytokine gene expression in T helper subsets. *J. Immunol.* 152, 77.
- Openshaw, P., Murphy, E.E., Hosken, N.A., Maino, V., Davis, D., Murphy, K., O'Gara, A., 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med.* 182, 1357.
- Pinkston, P., Bitterman, P.B., Crystal, R.G., 1983. Spontaneous

- release of IL-2 by lung T-lymphocytes in active pulmonary sarcoidosis. *New Engl. J. Med.* 308, 793.
- Prior, C., Knight, R.A., Herold, M., Ott, G., Spiteri, M.A., 1996. Pulmonary sarcoidosis: patterns of cytokine release in vitro. *Eur. Respir. J.* 9, 47.
- Robinson, B.W.S., McLemore, T.L., Crystal, R.G., 1985. Gamma interferon is spontaneous released by alveolar macrophages and lung T lymphocytes in patients with pulmonary sarcoidosis. *J. Clin. Invest.* 75, 1488.
- Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., Corrigan, C., Durham, S.R., Kay, A.B., 1992. Predominant T_{H2}-like bronchoalveolar T-lymphocyte population in atopic asthma. *New Engl. J. Med.* 326, 298.
- Romagnani, S., 1994. Lymphokine production by human T cells in disease states. *Ann. Rev. Immunol.* 12, 227.
- Sander, B., Andersson, J., Andersson, U., 1991. Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol. Rev.* 119, 65.
- Schmitz, J., Assenmacher, M., Radbruch, A., 1993. Regulation of T helper cell cytokine expression: Functional dichotomy of antigen-presenting cells. *Eur. J. Immunol.* 23, 191.
- Tang, M.L.K., Varigos, G., Kemp, A.S., 1994. Reduced interferon-gamma (IFN-gamma) secretion with increased IFN-gamma mRNA expression in atopic dermatitis: evidence for a post-transcriptional defect. *Clin. Exp. Immunol.* 97, 483.
- Van der Meide, P.H., Dubbeld, M., Schellekens, H., 1985. Monoclonal antibodies to human immune interferon and their use in a sensitive solid-phase ELISA. *J. Immunol. Methods* 79, 293.
- Van Neerven, R.J.J., Van 't Hof, W., Ringrose, J.H., Jansen, H.M., Aalberse, R.C., Wierenga, E.A., Kapsenberg, M.L., 1993. T cell epitopes of house dust mite major allergen *Der p II*. *J. Immunol.* 151, 2326.
- Walker, C., Bode, E., Boer, L., Hansel, T.T., Blaser, K., Virchow, J.-C. Jr., 1992. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 146, 109.
- Walker, C., Bauer, W., Braun, R.K., Menz, G., Braun, P., Schwarz, F., Hansel, T.T., Villiger, B., 1994. Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. *Am. J. Respir. Crit. Care Med.* 150, 1038.
- Warren, C.J., Howell, W.M., Bhambhani, M., Cawley, M.I.D., Smith, J.L., 1991. An investigation of T-cell subset phenotype and function in the rheumatoid synovium using in situ hybridization for IL-2 mRNA. *Immunology* 72, 250.
- Ying, S., Durham, S.R., Corrigan, C.J., Hamid, Q., Kay, A.B., 1995. Phenotype of cells expressing mRNA for TH2-type (interleukin-4 and interleukin-5) and TH1-type (interleukin-2 and interferon gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal control subjects. *Am. J. Respir. Cell Mol. Biol.* 12, 477.
- Zangrilli, J.G., Shaver, J.R., Cirelli, R.A., Cho, S.K., Garlisi, C.G., Falcone, A., Cuss, F.M., Fish, J.E., Peters, S.P., 1995. sVCAM-1 levels after segmental antigen challenge correlate with eosinophil influx, IL-4 and IL-5 production, and the late phase response. *Am. J. Respir. Crit. Care Med.* 151, 1346.