

Assessment of T lymphocyte cytokine production in induced sputum from asthmatics: a flow cytometry study

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Summary

Background Asthma results from a bronchial inflammation in which Th2 lymphocytes play a pivotal role, as shown in invasive bronchial biopsies and broncho-alveolar lavages. Induced sputum (IS) is a non-invasive method of recovery of bronchial cells, which can be repeated in the same patients. However, lymphocyte activation has not been studied in IS to date, because of the low number of T cells recovered. Herein we took advantage of flow cytometry, a method suitable for the study of small cell populations, to assess T cell cytokine production in IS.

Objectives (1) To assess induced sputum T cell cytokine production by flow cytometry in asthmatic subjects and controls. (2) To compare the T cell cytokine production between symptomatic and non-symptomatic asthmatics.

Methods Thirteen asthmatics and 19 controls were included. Sputum was induced by a hypertonic saline. Sputum cells were stimulated and intracellular IL-13 and IFN- γ were detected in T cells by flow cytometry.

Results Stimulation induced an increase of IL-13 and IFN- γ production by T cells. This increase was higher in asthmatics. IL-13-producing T cells were increased in asthmatics after stimulation. In symptomatic asthma, IFN- γ -producing T cells were in higher proportion than in controlled asthma.

Conclusion IS T cell cytokine production indicates a basic Th2 bias in asthma, accompanied during symptoms by a Th1-like activation. These results open the field for longitudinal studies of the variation of T cell activation in asthma.

Keywords asthma, flow cytometry, IFN- γ , IL-13, induced sputum, Th1, Th2

Submitted 13 November 2002; revised 17 March 2003; accepted 9 June 2003

Introduction

Studies conducted during the last decade established clearly that bronchial inflammation in asthma was characterized by the presence of IL-4- and IL-5-secreting T cells in bronchi, i.e. Th2 cells, thought to orchestrate the inflammatory reaction leading to eosinophil accumulation and IgE production within the airways [1, 2]. However, this Th2 paradigm in asthma was built on results obtained from bronchoalveolar lavages (BAL) and bronchial biopsies. The use of these materials in asthma is limited in that they are invasive and cannot be easily repeated in the same patients. Indeed, asthma oscillates from a quiet state in which a basic inflammation is found but no symptoms are felt, to an

active state in which inflammation varies while symptoms rise. In this view, non-invasive methods are required, which would permit the study in the same patients of the variation of inflammation according to the presence of symptoms.

Induced sputum (IS) is a recently generalized non-invasive method of recovering bronchial cells [3–5]. It is a promising approach of inflammation in asthma in that it can be repeated in a same patient at successive occasions and thus offer a longitudinal view of the asthmatic inflammation, more relevant to the variation in time of the disease activity. Recent studies described the cellular populations recovered by IS in asthma, and have notably showed that during asthma exacerbation, sputum eosinophilia increases before the occurrence of functional modifications and symptoms [6]. By contrast, the T lymphocyte activation was not studied to date in IS, essentially because of the scarcity of T cells in this material.

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In this work, we took advantage of flow cytometry, a method suitable for the study of small cell populations, to evaluate the possibility of assessing T cell cytokine production in IS at the single cell level [7, 8].

Subjects and methods

Subjects

Thirty-two subjects were included. Thirteen (seven males, six females, mean age 41.8 ± 5.4 years) were patients referred to the allergy clinic for a history compatible with asthma. Nineteen (seven males, 12 females, mean age 35.7 ± 2.3 years) were healthy controls. For each subject, a metacholine challenge, skin prick tests and IS were performed. The ethical committee of Marseille-II approved the protocol, and each subject gave an informed consent. Patients were not included if they were smokers, pregnant, had displayed an infectious episode or taken systemic corticosteroids or antihistamines during the last month. Inhaled treatment was not discontinued for ethical considerations.

The diagnosis of asthma was confirmed on the basis of a history of dyspnoea and wheezes, with a reversible airflow obstruction or a positive methacholine challenge test. Reversible airways obstruction was characterized by a 20% increase in forced expiratory volume in 1 s (FEV₁) after the inhalation of 200 µg of albuterol. Methacholine challenge tests were performed when spirometric data were normal. Cumulative doses of methacholine were administered through a dosimeter (ME-FAR dosimeter, Elletromedically, Brescia, Italy), specific airway resistance (S_{Raw}) and FEV₁ measurements were made after each dose in an 830 L constant body plethysmograph (model Master Lab Jaeger, Wurzburg, Germany). Bronchial hyper-reactivity was defined by a 100% increase of S_{Raw} at 200 µg of metacholine or less. The PD₂₀ (dose of metacholine inducing a decrease of 20% of the FEV₁) was determined for each positive test.

The severity of asthma was graded from I to IV (intermittent to severe persistent) according to the GINA guidelines [9]. Active asthma was defined arbitrarily on the need of at least two puffs of rescue medication for asthma symptoms, or the presence of at least one nocturnal awakening for asthma symptoms during the last week.

The control group consisted of healthy volunteers without previous history of asthma or allergic disease. The absence of asthma was confirmed in these subjects by the stability of FEV₁ and S_{Raw} after the inhalation of a total dose of 500 µg of metacholine.

Induced sputum

IS was obtained by the method described by Pizzichini et al. [5]. Asthmatics, but not controls, inhaled 200 µg of salbutamol to prevent bronchoconstriction during induction. The induction was performed by using an aerosol of hypertonic saline. The aerosol was generated by an ultrasonic nebulizer (Systam, Villeneuve sur Lot, France). Increasing concentrations of saline were inhaled (3%, 4%, 5%) for 7 min each through a mouthpiece. After each inhalation period, an FEV₁ was measured for safety, subjects were asked to rinse their mouth with water and swallow it and blow their nose to reduce contamination of the sputum specimen with saliva and

post-nasal drip, and to try to cough sputum into a sterile container.

Sputum treatment and examination

The sputum samples were treated immediately. The specimen was poured into a Petri dish. The Petri dish was placed on a black sheet of paper to optimize the contrast, and all visible portions of sputum were carefully selected, carried between two needles into a preweighed tube and weighed. Dithiotreitol (DTT) (USB, Cleveland, OH, USA) 0.1% freshly prepared was added in a volume (in µL) equal to three times the weight of sputum (in mg). The same volume of phosphate-buffered saline (PBS) was added. The mixture was then moved on a row for 20 min and filtered. Cells were centrifuged and resuspended in 500 µL of PBS. Five microlitres of suspension was used for assessment of viability with Trypan blue, and total cell count. 300 µL was used for cytopspins. Cytopspins were stained by the May–Grunwald–Giemsa method to allow a differential cell count.

Sputum cell culture

Cell culture was performed at 37 °C in an atmosphere containing 5% CO₂. Cells were resuspended at a concentration of 10⁶/mL, and cultured in 96-well plates in a volume of 50 µL in the presence or absence of 100 ng/mL of phorbol 12-myristate acetate (PMA) (Sigma Aldrich, St Quentin, France) and 2 µg/mL of ionomycin (Sigma Aldrich), and in the presence of brefeldin (20 µg/mL) (Sigma Aldrich) and monensin (2 µmol/L) (Sigma Aldrich). After 3 h of culture, cells were harvested, centrifuged, washed and resuspended in PBS. Optimal culture conditions were determined in preliminary experiments.

Immunostaining

Cells were incubated with 10 µL of PE-Cy5-coupled anti-CD3 monoclonal antibody (mAb) (IgG1, clone UCHT1, Immunotech, Beckman-Coulter, Marseilles, France) for 15 min in the dark. Cells were then fixed and permeabilized by using the IntraPrep[®] reagents (Immunotech) as indicated by the manufacturer. Cells were then incubated for 15 min with 40 ng of FITC-coupled anti-IFN-γ mAb (clone 4S B3, IgG1, Pharmingen, San Diego, CA, USA) and with 20 µL of PE-coupled anti-IL-13 mAb (clone JES10-5A2, IgG1, Biosource, Clinisciences, Montrouge, France). FITC- and PE-conjugated control isotypes (IgG1, clone 679.1 Mc7, Immunotech) were used in control experiments. After a wash in PBS, cells were centrifuged and resuspended in 250 µL of PBS 0.5% formaldehyde. Optimal concentrations of antibodies were determined in preliminary experiments.

Intracellular cytokine detection by flow cytometry

Flow cytometric data were immediately acquired on a Coulter EPICS XL/MCL flow cytometer (Beckman Coulter, Miami, FL, USA), using XL II TM software. Dead cells, monocytes and polymorphonuclear cells were excluded by forward and side scatter gating. Acquisition was gated on the CD3⁺ cells, and the whole sample T cell content was acquired. Statistical markers were set using the negative

control as reference. Results are expressed as the percentage of IFN- γ - or IL-13-expressing CD3⁺ cells.

Statistical Analysis

Because of the limited number of patients, non-parametric tests were used. A *P*-value of 0.05 or lower was considered as significant. All the analyses were performed using the Sigmatat[®] Software for Windows (SPSS Inc., Chicago, IL, USA).

Characteristics of subjects and differential cell counts were compared between groups by using the Mann–Whitney *U*-tests. Age, FEV₁ and FVC are expressed as mean $\pm$ the standard error to the mean (SEM). Differential cell counts were expressed as median and interquartile ranges (25%–75%).

We then analysed the sputum T lymphocyte activation. Results are expressed as median and interquartile ranges (25–75%). The proportions of T cells spontaneously producing IL-13 or IFN- γ were compared to those of T cells producing the same cytokines upon stimulation by using a paired Wilcoxon test. The Mann–Whitney *U*-test was used to compare proportions of cytokine-producing cells between patients and controls and between active and inactive asthmatics. To assess the Th2 paradigm, we compared the IL-13 producing T cells to IFN- γ producing T cells ratios (IL-13/IFN- γ) between asthmatics and controls. Correlations between variables were performed using a Spearman's test.

Results

Characteristics of the subjects

Eight asthmatics and eight controls were atopic (at least one positive skin prick test to a common environmental allergen). Among atopic asthmatics, two were sampled during the pollinic season relevant to their sensitizations. One was sensitized to parietaria pollen and sampled in April, and the other displayed positive test for grass pollen and was sampled in May. These two subjects were taking rescue medication every day at the time of sampling. Among atopic controls, two were sampled during a pollen season relevant to their sensitizations. One displayed positive prick test for cypress allergy and was sampled in January. The second was sensitized to grass pollen and sampled in July. None of them displayed allergic symptoms. In all other cases, subjects were sensitized only to house dust mites or were sampled out of the relevant pollen season.

One asthmatic was intermittent (grade I) and was not under inhaled steroids, 1 was mild persistent (grade II) and was treated by 500 μ g/day of beclometasone, 10 were moderate persistent (grade III) and were treated by 500 μ g/day (*n* = 6) or 1000 (*n* = 4) of beclometasone or equivalent and one was severe persistent (grade IV) and received 500 μ g/day of fluticasone. Six asthmatics were considered as active asthmatics, among which five were treated by 500 μ g/day of beclometasone and one by 1000 μ g/day. The dose of inhaled steroids was not different between active and controlled asthmatics. The functional characteristics of subjects are indicated in Table 1.

Table 1. Characteristics of the subjects

	Controls	Asthmatics
Age (years $\pm$ SEM)	35.9 $\pm$ 2.5	45.7 $\pm$ 4.3
FEV ₁ (% of predictive value)	110.1 $\pm$ 3.7	89.3 $\pm$ 5.5
FVC (% of predictive value)	112.0 $\pm$ 3.9	100.5 $\pm$ 6.5
FEV ₁ /FVC	83.9 $\pm$ 1.7	68.5 $\pm$ 3.4
Log PD20	NA	2.3 $\pm$ 0.1
Atopy (<i>n</i>)	8	8

NA, not applicable.

Table 2. Median and interquartile ranges (25–75%) of sputum total and differential cellular counts

	Controls	Asthmatics
Cells recovered ($\times 10^6$ /mL)	1.02 [0.72–2.49]	1.29 [0.66–1.98]
Lymphocytes (%)	2.5 [1–7]	2 [1–3]
Polymorphonuclear eosinophils (%)	0 [0–0]	0 [0–10.75]
Polymorphonuclear neutrophils (%)	42.5 [28–68]	36 [24.75–83.5]
Macrophages (%)	54 [31–64]	25 [9.75–68.75]

Sputum cell counts

Sputum cell counts were compared between asthmatics and controls (Table 2). No difference was found in total cell count, lymphocyte, polymorphonuclear neutrophils and eosinophils and macrophages between the two groups. 5/13 asthmatics and none of the controls displayed a sputum eosinophilia above 3%, but the difference was not significant.

Spontaneous T cell production of IL-13 and IFN- γ

The proportion of cytokine-producing T cells was analysed without stimulation in asthmatics and controls. More T lymphocytes produced IL-13 within the asthmatic group (0.33%, [0.05–0.46]) than in controls (0% [0–0.378]), but the difference was not significant (*P* = 0.289). T cells spontaneously producing IFN- γ were found in the same proportion in each group (3.18% [2.41–4.483] in asthmatics vs. 3.17% [2.31–5.68] in controls). The IL-13/IFN- γ ratio was not significantly different between groups (not shown).

Ex vivo stimulation of sputum T cells

The proportion of cytokine-producing cells was compared between stimulated and non-stimulated T cells. The number of IL-13 and IFN- γ -producing T cells significantly increased in both groups after stimulation by PMA and ionomycin (Table 3). The increase in the percentage of IFN- γ -producing T cells was higher in asthmatics than in controls, but the difference was not significant. The increase in IL-13-producing T cells was significantly higher in asthmatics as compared to controls (Table 4). In asthmatics, but not in controls, the proportion of cytokine-producing T cells was positively correlated to the proportion of lymphocytes found at the differential cell count performed on coloured cytopins.

Table 3. *Ex vivo* stimulation of T cells; median and interquartile ranges (25–75%) of percentages of IL-13- and IFN- γ -producing cells without and with non-specific stimulation

	IL-13-producing T cells (% of T cells)			IFN- γ – producing T cells (% of T cells)		
	Medium	PMA-ionomycin	<i>P</i> -value	Medium	PMA-ionomycin	<i>P</i> -value
Controls	0 [0–0]	0.27 [0.02–0.52]	0.049	3.17 [2.31–5.68]	4.7 [2.83–7.19]	0.031
Asthmatics	0.33 [0.05–0.46]	0.96 [0.38–1.06]*	0.031	3.18 [2.41–4.48]	5.85 [4.07–7.53]	0.005

*Statistical significant difference between asthmatics and controls ($P = 0.032$).

Table 4. Increase in cytokine-producing cells upon non-specific stimulation; median and interquartile ranges (25–75%) of differences between cytokine-producing T cells before and after non-specific stimulation.

	Increase in IFN- γ -producing T cells	Increase in IL-13-producing T cells
Controls	0.72 [–0.133–2.617]	0.16 [0–0.255]
Asthmatics	1.5 [0.638–4.148]	0.63 [0.25–0.722]
<i>P</i> -value	0.241	0.032

This result was statistically significant for IFN- γ only (IL-13: $R = 0.69$, $P = 0.07$; IFN- γ : $R = 0.54$, $P = 0.05$).

T cell production of IL-13 and IFN-gamma upon stimulation

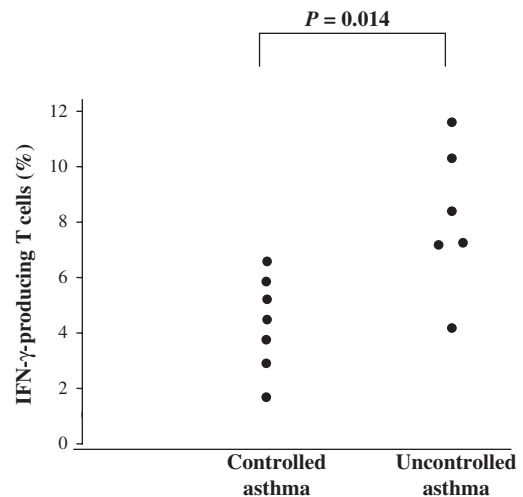
Upon stimulation, the proportion of IFN- γ -producing T cells was higher in asthmatics than in controls, but the difference was not significant. The proportion of IL-13-producing T cells was significantly higher in asthmatic sputum after stimulation (Table 3). The IL-13/IFN- γ ratio was significantly higher in asthma (0.114 [0.0882–0.395] vs. 0.0506% [0.0029–0.0943], $P = 0.029$).

T cell activation according to functional parameters, atopy, asthma severity and activity

No difference was found in cytokine T cell production according to FEV₁, FVC, PD20, atopic status and grades of severity. In contrast, the proportion of cytokine-producing T cells varied according to the activity of the disease. Among the 13 asthmatics, six needed at least two puffs of rescue medication in the last 7 days. The proportion of IL-13- and IFN- γ -producing T cells was increased in this subgroup compared to controlled asthmatics, but the difference was significant only for IFN- γ (IL-13: 1.08% [0.99–2.88], $P = 0.11$, IFN- γ : 7.82% [7.17–10.3], $P = 0.014$) (Fig. 1).

Discussion

IS is a non-invasive method of recovering bronchial cells largely used in studies of bronchial asthmatic inflammation. Most of these studies have focused on sputum eosinophilia [3, 4]. Indeed, this parameter has proved to be reproducible in asthmatic patients and reliable to various features of asthma [10]. Sputum eosinophilia is increased in asthma as compared to controls. Furthermore, it is higher in symptomatic asthma

**Fig. 1.** Sputum T cell cytokine production according to asthma activity. Asthmatics needing more than two puffs of rescue medication during the last week were compared to asthmatics without any need for rescue medication for their sputum T cell cytokine production. Sputum cells were cultured for 3 h in the presence of PMA and ionomycin. Cells were recovered and stained by anti-CD3, anti-IFN- γ monoclonal antibodies coupled to fluorochromes. Percentages of IFN- γ -producing T cells among the total of T cells were compared between groups by the Mann–Whitney *U*-test.

[11]. The increase of sputum eosinophilia during steroid withdrawal precedes modifications of functional measurements and symptoms of exacerbation [6]. Recently, sputum eosinophilia was reported as related to asthma severity [12]. It is decreased by systemic steroids, and also by inhaled steroids when given at 800 μ g/d of beclometasone or equivalent or more [13]. This last point is probably why we did not find any elevation of sputum eosinophilia in the present study, as most asthmatic patients were treated by inhaled steroids.

Bronchial eosinophilia is known as a relatively specific feature of asthma, also present in eosinophilic bronchitis [14]. In asthma, it is considered as a consequence of a Th2 cell activation, via notably the secretion of IL-5 and IL-13 within airways [15, 16]. Indeed, IL-5 and IL-13 are potent activators of eosinophils. Many studies in the last decade have confirmed in humans the prominent role of Th2 cells in asthma pathophysiology. The expression of IL-4, IL-13 and IL-5 genes was increased in BAL and bronchial biopsies from asthmatics [1, 2, 17]. In addition, it was shown that T lymphocytes were indeed responsible for the production of Th2 cytokines in asthma, albeit other cell types such as eosinophils themselves were IL-5 and IL-13 producers [18]. As showed in various studies, cells recovered by IS originate from a distinct airways compartment than those from BAL or

bronchial biopsies [19–22]. Thus, cytokine results obtained in the latter cannot be extrapolated to IS. Very few works were published concerning the IS T cell cytokine production. IL-5 was assayed by ELISA in IS supernatants and was found elevated in asthma compared to controls, but IL-5 concentrations were frequently below the detection threshold and the cellular source was uncertain [23]. Hamzaoui et al. [24] recently detected mRNA of various cytokines by RT-PCR in induced sputum, but again the cellular source of the cytokines could not be assessed. Previously, Olivenstein et al. [25] had shown by *in situ* hybridization the elevation of IS IL-4 and IL-5 gene expression in CD3⁺ cells from asthmatics, but the proteins themselves were not detected. Thus, we wanted to assess the Th1/Th2 paradigm in IS by using flow cytometry, a method able to show the cytokines produced within their cellular source. In addition flow cytometry, compared to *in situ* hybridization, immunocytochemistry and RT-PCR, is a more suitable technique to visualize small cell populations, as all the individuals belonging to the cellular subset of interest are sorted together by the cytometer. T Cells can therefore be studied as a whole population [8]. The study of cytokine production by flow cytometry necessitates in most cases an *ex vivo* stimulation to enhance the production of cytokine per cell while brefeldin and monensin block secretion. A short time of culture under PMA and ionomycin stimulation, although not TCR mediated respects the profile of cytokines being synthesized *in vitro* before sampling [7]. The increase of cytokine expression after stimulation proved that sputum lymphocytes could be cultured. However, the number of cytokine-positive cells was low as compared to blood. This could be due to an effect of sputum treatment. Indeed, it was shown that DTT treatment reduces the fluorescence of stained surface antigens, so that it could also reduce the level of expression of intracellular antigens such as cytokines [26]. The low number of cytokine-positive cells could also be related to a lower capacity of sputum cells to produce cytokines, perhaps due to inhaled steroids. Recently, it was shown that a high number of IS T cells displayed the phenotype of intraepithelial lymphocytes [27]. Little is known about the cytokine production capacities of this subset of T cells. The reproducibility of the results could not be tested inasmuch as the complete sputum sample was used for cell counts and flow cytometry and as each patient produced a single sample. However, the intraassay reproducibility is indirectly reflected by the fact that in each sample the number of cytokine-positive cells was higher in the stimulated than in the non-stimulated aliquot. Further studies with repeated sputum in a group of patients is required to assess the intrapatient reproducibility of the method.

We have demonstrated by flow cytometry the presence of a prominent Th2 cell activation in IS from asthmatics, compared to control subjects. We chose IL-13 and IFN- γ , as these two cytokines are very representative of Th2 and Th1 activation, respectively. IL-13-producing T cells and the ratio of IL-13-producing cells to the IFN- γ -producing cells was higher in asthmatics than in controls. Moreover, we have shown that T cells from asthmatic sputum displayed a higher capacity than controls to produce IL-13, but not IFN- γ upon PMA and ionomycin stimulation.

Unexpectedly, the comparison between symptomatic and non-symptomatic asthmatics revealed that in active asthma,

both IL-13- and IFN- γ -producing T cells were increased as compared to controlled asthma. The increase of IL-13 is concordant with previous studies that showed higher bronchial eosinophilia among symptomatic asthmatics [11]. However, the increase of IFN- γ -producing T cells is more questionable. It is not attributable to inhaled steroid treatment as most asthmatics from both groups were treated. IFN- γ was found increased in the past in sera from severe asthmatics [28, 29]. At the single cell level, Krug et al. [30] had found an increase in IFN- γ production by T cells recovered from asthmatic BAL. More recently, we have found in blood that IFN- γ was inversely related to atopy but positively correlated to asthma [31]. Moreover, we have shown in asthmatic blood the presence of a CD8⁺ population of T cells producing IFN- γ that was correlated to bronchial hyper-reactivity and asthma severity. These results have been confirmed by others and extended to CD4⁺T cells [32]. Thus, various studies indicate that in addition to the Th2 activation, a Th1 cell activation is present in asthma [33]. Our present findings extend these data in showing that IFN- γ -producing cells are present in sputum from asthmatics and that these cells are related to the presence of symptoms. Some attempt was made to determine if IFN- γ - and IL-13-producing T cells belonged to the CD8⁺ subset, but the number of cytokine-producing CD8⁺ cells was too low to allow statistical analysis (not shown). This indirectly suggests that the majority of cytokine-producing T cells belonged to the CD4⁺ subset. The presence of a Th1 activation related to symptoms questions the relevance of therapeutic strategies based on targeting the Th2 activation only. In this view, the recent failures of clinical trials with anti-IL-5 or IL-12 are noteworthy [34, 35].

Taken together, our results suggest that a basic Th2 bias exists in asthma, but that during symptomatic periods a Th1 activation accompanies the increase of Th2 cytokine production. The basic Th2 bias is not explained by atopy inasmuch as atopic subjects were as frequent in the asthmatic as in the control group.

In conclusion, this study shows that sputum T cell cytokine production can be studied, even after *ex vivo* culture. It opens the field of longitudinal studies of IS in asthmatics, in which T cell activation will be considered according to symptoms.

Acknowledgements

This work was achieved with help of grants from INSERM, Société Française d'Allergologie et d'Immunologie Clinique, Pneumologie-développement, Assistance Publique Hôpitaux de Marseille and Collège des Professeurs de Pneumologie.

References

- 1 Robinson DS, Hamid Q, Ying S et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; 326:298–304.
- 2 Humbert M, Durham SR, Ying S et al. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and non-atopic asthma: evidence against "intrinsic" asthma being a distinct immunopathological entity. *Am J Respir Crit Care Med* 1996; 154:1497–504.

- 3 Pin I, Gibson PG, Kolendowicz R et al. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992; 47:25–9.
- 4 Boniface S, Donati Y, Romanet-Manent S, et al. L'analyse de l'expectoration induite dans l'asthme permet une nouvelle approche de l'inflammation. *Rev Mal Respir* 2002; 19:747–59.
- 5 Pizzichini MMM, Popov TA, Efthimiadis A et al. Spontaneous and induced sputum to measure indices of airway inflammation in asthma. *Am J Crit Care Med* 1996; 154:866–9.
- 6 Magnan A, Lim S, Barnes PJ. Changes in sputum eosinophils predict loss of asthma control. *Am J Respir Crit Care Med* 2000; 161:64–72.
- 7 Prussin C, Metcalfe DD. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Meth* 1995; 188:117–28.
- 8 Magnan A. La cytométrie en flux, les petites populations cellulaires et l'allergie. *Rev Mal Respir* 2000; 17:619–21.
- 9 NHLBI/WHO Workshop Report 2002. NIH Publication 02-3659.
- 10 Holz O, Richter K, Jörres RA, Speckin P, Mücke M, Magnussen H. Changes in sputum composition between two inductions performed on consecutive days. *Thorax* 1998; 53:83–6.
- 11 Pizzichini E, Pizzichini MMM, Efthimiadis A et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and phase-fluid measurements. *Am J Respir Crit Care Med* 1996; 154:308–17.
- 12 Ten Brinke A, Zwinderman AH, Sterk PJ, Rabe KF, Bel EH. Factors associated with persistent airflow limitation in severe asthma. *Am J Respir Crit Care Med* 2001; 164:744–8.
- 13 Jatakanon A, Kharitonov S, Lim S, Barnes PJ. Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. *Thorax* 1999; 54:108–14.
- 14 Gibson PG, Fujimura M, Niimi A. Eosinophilic bronchitis: clinical manifestations and implications for treatment. *Thorax* 2002; 57:178–82.
- 15 Menzies-Gow A, Robinson DS. Eosinophils, eosinophilic cytokines (interleukin-5), and antieosinophilic therapy in asthma. *Curr Opin Pulm Med* 2000; 33–8.
- 16 Wills-Karp M. IL-12/IL-13 axis in allergic asthma. *J Allergy Clin Immunol* 2001; 107:9–18.
- 17 Naseer T, Minshall EM, Leung DY et al. Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy. *Am J Respir Crit Care Med* 1997; 155:845–51.
- 18 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.
- 19 Pizzichini E, Pizzichini MM, Kidney JC et al. Induced sputum, bronchoalveolar lavage and blood from mild asthmatics: inflammatory cells, lymphocyte subset, and soluble markers compared. *Eur Respir J* 1998; 11:828–34.
- 20 Keatings VM, Evans DJ, O'Connor BJ, Barnes PJ. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. *Thorax* 1997; 52: 372–4.
- 21 Fahy JV, Wong H, Liu J, Boushey HA. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. *Am J Respir Crit Care Med* 1995; 152:53–8.
- 22 Grootendorst DC, Sont JK, Willems LN et al. Comparison of inflammatory cell count in asthma: induced sputum versus bronchoalveolar lavage and bronchial biopsies. *Clin Exp Allergy* 1997; 27:769–79.
- 23 Kelly MM, Leigh R, Carruthers S et al. Increased detection of interleukin-5 in sputum by addition of protease inhibitors. *Eur Respir J* 2001; 18:685–91.
- 24 Hamzaoui A, Brahim MB, Zhioua A, Ayed K, Hamzaoui K. Inflammatory response in induced sputum mononuclear cells from patients with acute exacerbation of asthma. *Mediators Inflamm* 2000; 9:147–53.
- 25 Olivenstein R, Taha R, Minshall EM, Hamid QA. IL-4 and IL-5 mRNA expression in induced sputum of asthmatic subjects: comparison with bronchial wash. *J Allergy Clin Immunol* 1999; 103:238–45.
- 26 Efthimiadis A, Pizzichini MMM, Pizzichini E, Dolovich J, Hargreave FE. Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiothreitol versus phosphate-buffered saline. *Eur Respir J* 1997; 10:1336–40.
- 27 Leckie MJ, Jenkins GR, Khan J et al. Sputum T lymphocytes in asthma, COPD and healthy subjects have the phenotype of activated intraepithelial T cells (CD69⁺ CD103⁺). *Thorax* 2003; 58:23–9.
- 28 Corrigan CJ, Kay AB. CD4 T lymphocyte activation in acute severe asthma. *Int Arch Allergy Appl Immunol* 1991; 94:270–1.
- 29 Cembrzynska-Nowak M, Szklarz E, Inglot AD, Teodorczyk-Injeyan JA. Elevated release of tumor necrosis factor- α and interferon- γ by bronchoalveolar leukocytes from patients with bronchial asthma. *Am Rev Respir Dis* 1993; 147:291–5.
- 30 Krug N, Madden J, Redington AE et al. T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am J Respir Cell Mol Biol* 1996; 14:319–26.
- 31 Magnan AO, Mely LG, Camilla CA et al. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN- γ -producing CD8(+) T cells in asthma. *Am J Respir Crit Care Med* 2000; 161:1790–6.
- 32 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.
- 33 Salvi SS, Babu KS, Holgate ST. Is asthma really due to a polarized T cell response toward a helper T cell type 2 phenotype? *Am J Respir Crit Care Med* 2001; 164:1343–6.
- 34 Leckie MJ, ten Brinke A, Khan J et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000; 356:2144–8.
- 35 Bryan SA, O'Connor BJ, Matti S et al. Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000; 356:2149–53.