ERJ Express. Published on November 1, 2006 as doi: 10.1183/09031936.00060205

Eotaxin 2 in sputum cell culture to evaluate asthma inflammation

Marcos Eduardo Scheicher[1]

Mauro Martins Teixeira[2]

Fernando Queiroz Cunha[3]

Antônio L. Teixeira Jr[4]

João Terra Filho[1]

Elcio Oliveira Vianna[1]

from

[1]Department of Medicine, University of S. Paulo Medical School at Ribeirão Preto.
[2]Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais.
[3]Department of Pharmacology, University of S. Paulo Medical School at Ribeirão Preto.
[4]Department of Internal Medicine, Medical School, Federal University of Minas Gerais, Brazil.

Corresponding author:
Elcio Oliveira Vianna
Pulmonary Division - Department of Medicine
University of S. Paulo Medical School at Ribeirão Preto.
Av. Bandeirantes, 3900, Ribeirão Preto, SP, 14048-900, Brazil.
Phone: 55-16-3602 2706; Fax: 55-16-3633 6695; E-mail: evianna@uol.com.br

Supported by grants from the S. Paulo State Government (FAPESP) - Brazil, Grants 02/02194-2 and 98/10382-6.

Running head: Eotaxin 2 in sputum cell culture in asthma.

Abstract

We aimed to elucidate whether culture of cells recovered from induced sputum may represent a suitable model to evaluate cytokines and chemokines production by airways inflammatory cells.

Sputum induction was performed in 21 normal subjects and 30 asthmatic patients: 21 were taking inhaled corticosteroid and 9 were steroid-naive asthmatics evaluated before and after 14-day treatment with oral prednisone (40 mg/day). The supernatant of lysed and centrifuged sputum and the supernatant of sputum cell culture were analysed. TNF-α, IL-8 (CXCL8), IL-1β, IL-13, and eotaxin 2 (CCL24) concentrations were determined by specific ELISA.

Eotaxin 2 production by cell culture was higher in the asthma group (131 ± 108 pg/ml) than in the control group (36 ± 41 pg/ml) and treatment with oral corticosteroid abolished this difference. In addition, reduction of eotaxin 2 levels by corticosteroid treatment was greater in cell culture (81.3% reduction) than in sputum (26.4%) ($p < 0.05$). There was correlation ($r = 0.74$; $p = 0.04$) between the decrease in eotaxin 2 production and the decrease in blood eosinophil number and between eotaxin 2 and eosinophils in sputum ($r = 0.56$; $p = 0.01$).

Eotaxin 2 may play an important role in asthma and response to corticosteroid treatment suggests that analysis of **sputum** cell culture is relevant as an inflammatory parameter.

Key words: asthma, sputum, inflammatory mediators, cell culture, steroid.

Introduction

Asthma is defined as a chronic lung disease with the following attributes: airflow obstruction that is reversible, either spontaneously or with treatment; airway inflammation and increased airways responsiveness to a variety of stimuli, including cold air, methacholine, hypertonic solution inhalation and exercise (1). The aetiology of asthma is complex and involves the interaction between genetic factors and environment. The vast majority of the data regarding the pathogenesis of asthma concentrate on atopic asthma and the imbalance between the Th1 and Th2 phenotypes (2). Further evidence of an inflammatory response in asthma is the presence of cells and mediators in bronchoalveolar lavage, sputum, and respiratory tissues. Eosinophil recruitment from peripheral blood into the airways is controlled by adhesion molecules and chemokines.

The chemokines are a group of chemotactic cytokines that have been subdivided into four subfamilies: CXCL, CCL, CL, and CX3CL (3,4). Members of the CCL branch include RANTES (CCL5), monocyte chemotactic proteins (MCP)-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), eotaxin 1 (CCL11), eotaxin 2 (CCL24) and eotaxin 3 (CCL26). RANTES, MCPs-3 and -4 are chemotactic for eosinophils, monocytes and lymphocytes, while eotaxins chemoattract eosinophils, basophils and Th2-lymphocytes (4). Because of their eosinophil chemotactic properties, these chemokines have attracted major attention in allergic inflammation (5). Studies conducted in adult asthmatics have shown that RANTES, MCPs-3 and -4, and eotaxins 1 and 2 are implicated in the asthmatic reactions (6-10).

In clinical practice, assessment of airway inflammation and effects of medication on inflammation is difficult. Subjective assessment of symptoms has often been found to be unsatisfactory for monitoring asthma severity (11). Measurements of levels of exhaled gases such as nitric oxide may be useful, but more data are needed to fully evaluate the

importance of these markers in assessing airways inflammation in asthma, especially because nitric oxide can be produced in large amounts in paranasal sinuses and stomach (11). Repeated bronchoscopic sampling is not feasible in large scale. The ability to study inflammation has changed considerably with the development of the induced sputum technique as a research and, increasingly, a clinical tool (12,13). Induced sputum was used for the first time in asthmatic patients in 1992, when Pin et al adapted the technique used until then for diagnosis of *Pneumocystis carinii* in patients with HIV (14). In recent years, sputum induction with hypertonic saline and subsequent processing have been refined as a non-invasive research tool providing important information about inflammatory events in the lower airways.

The technique of cell culture has been developed, especially, for blood cells in an attempt to reproduce *in vitro* what happens *in vivo*. Several authors have used the culture of blood cells to study asthma pathophysiology and responsiveness to treatments (15-17). However, very few studies employed culture of sputum cells (18-22). It is likely that cells from airways provide better information due to their proximity to the site of asthmatic inflammation. The aim of our study was to elucidate whether the culture of cells recovered from induced sputum may represent a suitable model to evaluate cytokine and chemokine production by airway inflammatory cells. Thus, we have assessed culture time (viability) and the production of inflammatory mediators from stimulated and non-stimulated, adherent and non-adherent cells in asthmatics and in healthy subjects. The response to corticosteroid treatment was also measured by analysis of sputum cell culture.

Material and methods

Subjects

Fifty-one subjects were included in the study. Thirty stable asthmatic patients (14 males, mean age 41.0 years) were recruited from the asthma clinic of the University Hospital of Ribeirão Preto, University of São Paulo. Twenty-one patients used regular inhaled corticosteroids (mean daily dose 409.5 ± 40.1 μg budesonide) and were studied in a single visit. This group was denominated asthma inhaled corticosteroid (IC) group. Nine uncontrolled, untreated asthmatics (on bronchodilator) were studied at two time-points: before (pre-treatment) and after (post-treatment) oral corticosteroid (OC) treatment for 14 days (prednisone 40 mg/day). These patients did not present acute asthma, they were scheduled for prednisone treatment independently of asthma severity, and the choice for this treatment was due to research purposes. The variables under study and blood eosinophil counts were determined in these patients before and after treatment. Twenty-one normal volunteers (six males, mean age 41.1 years) with forced expiratory volume in the first second (FEV_1) values of at least 90% of predicted formed a control group (Table 1). Smokers, pregnant women, subjects who had experienced a recent infectious episode or had taken systemic corticosteroids or antihistamines drugs during the last month were excluded from the patient and control groups.

The diagnosis of asthma was confirmed on the basis of a history of dyspnoea and wheezing, with a reversible airflow obstruction characterised by a 15% increase in FEV_1 after inhalation of 200-400 μg albuterol and/or a methacholine $PC_{20} < 8$ mg/ml (23). The study was approved by the Ethics Committee, University of S. Paulo Medical School at Ribeirão Preto. All volunteers gave informed consent to participate in the study.

Sputum induction and processing

For the sputum induction and processing, we applied the guidelines that are suggested by the Task Force on Induced Sputum of the European Respiratory Society (24,25). All subjects had an FEV_1 > 50% predicted. After pre-medication with 200-400 μg inhaled albuterol, sputum was induced by inhalation of a hypertonic saline (NaCl 4.5%) aerosol delivered by an ultrasonic nebulizer (Ultra-Neb 2000, DeVilbiss-Sunrise Medical, Somerset, PA, USA). Each subject inhaled the aerosol for 4 consecutive periods of 5 minutes for a total time of 20 minutes. For safety reasons, the peak expiratory flow (PEF) was monitored every 5 minutes and the induction stopped when the PEF fell to the critical value (a 10% fall from the basal value) or in case of severe symptoms. If a subject tolerated sputum induction for less than 20 minutes at pre-treatment visit, then the duration of sputum induction at post-treatment visit was kept the same as at pre-treatment visit. Subjects were told to spit saliva into one container periodically and sputum into another. The sputum was weighed and equal volume of 1 mg/ml dithiothreitol (GIBCO BRL, Grand Island, NY, USA) was added. The suspension was shaken in a vortex mixer for few seconds and incubated in a shaking water bath at 37ºC (150 cycles/min) for 15 minutes, with aspiration every 5 minutes for homogenisation. Centrifugation was performed at 750 g for 10 minutes. The supernatant was aspirated and stored. The cell pellet was resuspended in 1 ml RPMI-1640 medium containing 300 mg/l L-glutamine; 100 U/ml penicillin G; 100 μg/ml streptomycin sulfate (GIBCO BRL, Grand Island, NY, USA), and 10% calf serum. Total cell number and cell viability were determined by the Trypan blue exclusion method

in a Neubauer chamber and slides were prepared for differential cell counts by cytospin staining with Giemsa stain. The cell concentration was adjusted to 1 x 10^6 viable cells/ml.

Characteristics of sputum induction are shown in Table 1. Asthma IC group had higher total cell number and asthma OC group had lower duration of inhalation.

Cell culture

The cell suspension was then plated at 5 x 10^5 cells/well onto a 48-well plastic culture plate (Nunc Brand Products, Roskilde, Denmark) and allowed to adhere for 1 hour at 37º C/5% CO_2. After this adherence period, supernatant of every well was aspirated to remove cells, called non-adherent cells, and wells were washed twice with PBS at 37º C. Cells that were not removed, called adherent cells, were kept in the same wells, and 0.5 ml/well of medium was then added. The number and viability of non-adherent cell population were determined. These cells were re-plated: 1 x 10^5 viable cells/well, with 0.2 ml/well, in 96-well plastic culture plates (Nunc Brand Products, Roskilde, Denmark) and stimulated with phytohaemaglutinin (PHA), 10 μl/well (GIBCO BRL, Grand Island, NY, USA). Cells were incubated for 24, 48, 72 hours or 5 days at 37º C/5% CO_2.

To perform differential cell counts of the adherent cell culture, sputum cells were also plated on sterile chambers mounted on Permanox slides with cover (Chamber Slide System, Nalge Nunc International Corp., IL, USA). At the end of culture time, slides (with adherent cells) were detached from media chamber. For the non-adherent cells, at the end of culture time, cells were aspirated and slides were prepared by cytospin staining.

Time-response analysis

After completion of the culture time, cell viability was determined by the Trypan blue exclusion method and a time-response curve was plotted for adherent and non-adherent cells of asthmatics. The supernatant of cell culture was aspirated and frozen at -85º C until measurement of cytokines.

LPS response

The cells were cultured and incubated as described above. The adherent cells were stimulated with LPS (10 μl/well) at the following concentrations (in the culture): zero (negative control), 0.02 μg/ml, 0.002 μg/ml and 0.0002 μg/ml.

Detection of cytokines and chemokines

The concentrations of chemokines (eotaxin 2 and IL-8 [CXCL8]) and cytokines (TNF-α, IL-1β and IL-13) in induced sputum and cell culture supernatant of asthmatic patients and controls were measured by ELISA (R&D Systems, Minneapolis, Minn., USA, and Pharmingen, San Diego, Calif., USA).

Statistical analysis

Data concerning viability and inter-group comparisons are reported as means ± S.D. and were analysed statistically by Kruskal-Wallis test with Dunn post-test. Intra-group comparisons between stimulated and non-stimulated cells and of change (Δ) in eotaxin 2 production were performed by Wilcoxon matched-pairs test. Correlations between parameters were calculated by Spearman rank correlation. P-values ≤ 0.05 were considered to be statistically significant.

Results

Characterisation of cell culture

This first part of the analysis of cell culture was performed in asthmatic subjects (asthma IC group). Viability was high and similar for adherent and non-adherent cells up to 72 hours of culture. After 72 hours, the viability dropped significantly (Figure 1). TNF-α, IL-8 and IL-1β production by adherent cells reached the highest values at 48 hours of culture: 1417 ± 1128 pg/ml, 785 ± 321 pg/ml, and 94 ± 153 pg/ml, respectively. The production of TNF-α and eotaxin 2 by non-adherent cells was also highest at 48 hours. Therefore, this time point was used to monitor the production of inflammatory markers. Cytokines levels with and without stimulation at the highest concentration of LPS or PHA were not statistically different (**Table 2**). **Cytokines produced by adherent and non-adherent cells in the comparison of asthma IC and control groups are shown in Table 3**. The production of IL-13 by non-stimulated and stimulated non-adherent cells was below the detection limit of the assay.

Effects of oral corticosteroid treatment

Squamous cell percentage in induced sputum was 21.6 ± 5.0% in the control group, 19.9 ± 2.4% for the asthma OC group pre-treatment, 28.4 ± 5.1% post-treatment, and 12.3 ± 6.2% in the asthma IC group (non-significant differences). Differential cell counts of sputum, adherent and non-adherent cell cultures for all groups are shown in **Table 4**. Asthma IC group and asthma OC group at baseline had higher proportion of eosinophils in both sputum and non-adherent fraction. Higher percentage of eosinophils was also detected in the adherent cell culture, significantly in the asthma OC group at baseline.

The number of non-adherent cells at the end of 48 hours of culture was similar among groups ($p = 0.92$). Values for controls were $2.8 \pm 3.0 \times 10^4$ cells/ml, for patients treated with inhaled corticosteroids were $2.4 \pm 1.2 \times 10^4$ cells/ml, and for patients treated with oral corticosteroids were $2.3 \pm 1.0 \times 10^4$ cells/ml.

Eotaxin 2 production in culture of non-adherent cells (**Figure 2**) did not differ significantly between the control and asthma OC (post-treatment) groups ($p = 0.61$), but differed significantly between asthma IC and control groups ($p = 0.03$), as well as between asthma IC and asthma OC (post-treatment; $p = 0.04$). In the asthma OC group, values of eotaxin 2 concentration were 192 ± 102 pg/ml (pre-treatment) and 30 ± 30 pg/ml (post-treatment; $p = 0.05$). TNF-α production by non-adherent cells was 3638 ± 2835 pg/ml in the pre-treatment phase of asthma OC group and 2594 ± 2178 pg/ml in the post-treatment phase ($p > 0.05$).

Sputum eotaxin 2 levels correlated with cell culture eotaxin 2 production ($r = 0.67$; $p = 0.04$). The response to oral corticosteroid treatment was evaluated by calculating the difference between the levels of eotaxin 2 before and after prednisone treatment in induced sputum and in sputum cell culture (**Figure 3**). The response was greater in cell culture, 134 ± 80 pg/ml, than in induced sputum, 23 ± 33 pg/ml ($p = 0.04$).

The blood eosinophil count in the asthma OC group fell from the pre-treatment, $8.6 \pm 4.8\%$, to the post-treatment period, $1.5 \pm 1.6\%$ ($p = 0.002$). The pre-treatment blood eosinophil number correlated with eotaxin 2 production by cell culture ($r = 0.71$; $p = 0.02$). The response in cell culture eotaxin 2 production to steroid treatment correlated with the response in blood eosinophil number and sputum eosinophil number ($r = 0.74$; $p = 0.04$).

Sputum eosinophil number correlated with eotaxin 2 level in sputum ($r = 0.56$; $p = 0.01$), but not with eotaxin 2 production by cell culture.

Discussion

In this study, we have shown the possibility of evaluating *in vitro* the inflammatory status of asthmatic patients. We cultured sputum inflammatory cells of asthmatic and normal subjects. In the few studies that tried to keep sputum cells in culture to evaluate the inflammatory characteristics of asthma, sputum cells were cultured without fractionation (18-22). We separated cells in two fractions, adherent and non-adherent, and evaluated cytokines, chemokines, and cell composition in each fraction. In the culture of adherent cells, we detected TNF-α, IL-8, and IL-1β from both the asthma and control groups. In the culture of non-adherent cells, we detected eotaxin 1, eotaxin 2, and TNF-α. Of the measured mediators, eotaxin 2 appeared to show the greatest differences between the various groups. Indeed, eotaxin 2 production was higher in the asthma group compared to the control group and treatment with oral corticosteroid abolished this difference. In addition, reduction of eotaxin 2 levels by this treatment was greater in cell culture than in sputum.

This study was the first to evaluate and detect eotaxin 2 in sputum and sputum cell culture of asthmatic patients. The first eotaxin was discovered using a guinea pig model of allergic airway disease, characterised by marked lung eosinophilia (26,27). In humans, Mattoli et al showed that the numbers of cells expressing mRNA for eotaxin 1 correlated significantly with the number of eosinophils, bronchial hyperreactivity and symptom score in asthmatics (28). Moreover, increased eotaxin 1 protein has been detected in the BAL fluid of asthmatic subjects (29). According to Miotto et al, eotaxin 1 is selectively increased in Th2-mediated airway diseases, and this chemokine plays a central role in the pathogenesis of asthma (10). In addition to stimulating eosinophil chemotaxis, eotaxin 1

induces aggregation of eosinophils, an elevation in their intracellular calcium levels (29) and respiratory burst activity. Similarly to eotaxin 1, eotaxin 2 is known to be a potent eosinophil chemoattractant *in vitro* and *in viv*o (30-32). A study of the late-phase allergic reactions in the skin of human atopic subjects suggested that eotaxin 1 is involved in the early 6 hours recruitment of eosinophils while eotaxin 2 is involved in the later 24 hours infiltration of these cells (32).

Our findings demonstrate a relationship between eotaxin 2 and asthma. Eotaxin 2 production in cell culture correlated with eosinophil number in peripheral blood and eotaxin 2 concentration in sputum correlated with eosinophil number in sputum. Moreover, the decrease in eotaxin 2 production induced by systemic steroid treatment was associated with the decrease in eosinophil number in sputum and blood. In culture, eotaxin 2 levels of patients treated with inhaled corticosteroids were significantly higher than those of controls and of patients treated with oral corticosteroids. This may indicate that the inflammatory status of patients treated with regular inhaled corticosteroid remains high. The detected percentage of eosinophils in sputum corroborates this theory. In a study on mild asthma, Jatakanon et al evaluated the effect of differing doses of inhaled budesonide on airway inflammation markers (33). In that study, they showed a significant trend towards greater reduction in inflammatory markers with higher dose of budesonide (1600 μg/day), suggesting a dose-dependent effect of inhaled steroids on airway inflammation. The elevated concentration of eotaxin 2 found in the present study may have been due to the fact that most asthmatic patients were treated with a low dose inhaled corticosteroid. In contrast, there was no significant difference in eotaxin 2 levels between patients treated with oral corticosteroids and controls. The effectiveness of oral corticosteroids in the

treatment of chronic asthma probably results from a combination of anti-inflammatory actions (34), these being the most potent anti-inflammatory drugs used in treatment of asthma, which inhibit gene transcription of the majority of cytokines involved in asthmatic airway inflammation (35,36,37).

As regards the constitution of sputum cell cultures, at baseline, asthmatic patients had higher proportion of eosinophils in all fractions. In the post-treatment evaluation, those numbers of eosinophils fell to values that were not different from the control group values. The proportion of macrophages increased from the time of sputum collection to the end of cell culture period in both adherent and non-adherent fractions, which may represent cell proliferation in culture. Further studies are necessary to evaluate the mechanisms of cell number changes and to determine whether the decrease in eotaxin production reflects changes in cell number or suppression by steroid effect.

We have performed a series of spike and recovery experiments with eotaxin 2 (data not showed). Our results clearly demonstrate that the processing of samples with DTT diminishes by around 50 to 60% the recovery of the chemokine in sputum samples. It is of note, however, that the effect of DTT on the samples was linear in the range of 30-300 pg of eotaxin 2/ml of sputum. This is to mean that the effects of DTT on eotaxin 2 determination were similar in all of our samples and that the processing itself was not responsible for the differences observed between the various groups of patients. These data confirmed previous studies (38).

The interference of DTT on eotaxin 2 measurements may explain, at least partially, the advantage of cell culture supernatant over sputum supernatant. Sputum cell culture supernatant has no DTT and eotaxin 2 is produced *de novo*. Thus, the

assay to measure mediators in culture supernatant is free of the DTT interference, which may account for the superior performance of sputum cell culture in detecting response to patient treatment.

The cytokines and chemokines could be detected *in vitro* without any additional stimulus in the culture medium. The results demonstrated no significant difference in inflammatory markers with stimulation of the adherent and non-adherent cells with LPS or PHA, respectively. Our results are consistent with the cells being activated during their migration to the airway and agree with those of Bettiol et al (18). In contrast, Liu et al (19) had to add PHA in order to obtain detectable levels of IFN-γ in their sputum cell culture.

In conclusion, the present study demonstrates the usefulness of *in vitro* sputum cell generation of cytokines and chemokines as a suitable model to evaluate the airway inflammation observed in asthmatic patients. Our findings demonstrate a correlation between eotaxin 2 production in cell culture and eosinophil number in peripheral blood or sputum. In addition, there was a greater fall in eotaxin 2 levels measured in cell culture than in induced sputum following treatment. Taken together, our results suggest that analysis of **sputum** cell culture is sensitive for the detection of the effects of anti-inflammatory drugs and are consistent with an important role of eotaxin 2 in the pathophysiology of asthma.

References

1. Spahn J, Covar R, Stempel DA. Asthma: Addressing consistency in results from basic science, clinical trials, and observational experience. *J Allergy Clin Immunol 2002; 109:S490-502.*

2. Maddox L, Schwartz DA. The pathophysiology of asthma. *Annu Rev Med 2002; 53:477-498.*

3. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity 2000; 12:121–127.*

4. Teran LM. CCL chemokines and asthma. *Immunol Today 2000; 21:235–242.*

5. Rojas-Ramos E, Avalos AF, Perez-Fernandez L, Cuevas-Schacht F, Valencia-Maqueda E, Teran LM. Role of the chemokines RANTES, monocyte chemotactic proteins-3 and -4, and eotaxins-1 and -2 in childhood asthma. *Eur Respir J 2003; 22:310–316.*

6. Ying S, Meng O, Zeibecoglou K, et al. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), MCP-4, andC-C chemokine receptor 3 expression in bronchial biopsies from atopic and non-atopic (Intrinsic) asthmatics. *J Immunol 1999; 163:6321–6329.*

7. Teran LM, Noso N, Carroll M, Davies DE, Holgate S, Schroeder JM. Eosinophil recruitment following allergen challenge is associated with the release of chemokine RANTES into asthmatic airway**.** *J Immunol 1996; 57:1806–1812.*

8. Lamkhioued B, Renzi PM, Abi-Younes S, *et al.* Increase expression of eotaxin in broncho alveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J Immunol 1997; 159:4593–4601.*

9. Lamkhioued B, Garcia-Zepeda EA, Abi-Younes S, *et al.* Monocyte chemoattractant protein (MCP)-4 expression in the airways of patients with asthma. Induction in epithelial cells and mononuclear cells by proinflammatory cytokines. *Am J Respir Crit Care Med 2000; 162:723–732.*

10. Miotto D, Christodoulopoulos P, Olivenstein R, et al. Expression of IFN-γ –inducible protein; monocyte chemotactic proteins 1, 3, and 4; and eotaxin in TH1- and TH2-mediated lung diseases. *J Allergy Clin Immunol 2001; 107:664-670.*

11. Vignola AM, Rennard SI, Hargrave FE, et al. Future directions. *Eur Respir J 2002; 20:51s-55s.*

12. Kips JC, Inman MD, Jayaram L. The use of induced sputum in clinical trials. *Eur Respir J 2002; 20:47s-50s.*

13. Djukanovic R, Sterk PJ, Fahy JV, Hargreave FE. Standardized methodology of sputum induction and processing. *Eur Respir J 2002; 20:1s-2s.*

14. Pin I, Gibson PG, Kolendowicz R, et al. Use of sputum induced cell counts to investigate airway inflammation in asthma. *Thorax 1992; 47:25-29.*

15. Corrigan CJ, Bungre JK, Assoufi B, Cooper AE, Seddon H, Kay AB**.** Glucocorticoid resistant asthma: T-lymphocyte steroid metabolism and sensitivity to glucocorticoids and immunosuppressive agents**.** *Eur Respir J 1996; 9:2077-2086.*

16. Vianna EO, Westcott J, Martin RJ. The effects of upper respiratory infection on T-cell proliferation and steroid sensitivity of asthmatics. *J Allergy Clin Immunol 1998; 102:592-597.*

17. Kraft M, Vianna E, Martin RJ, Leung DY. Nocturnal asthma is associated with reduced glucocorticoid receptor binding affinity and decreased steroid responsiveness at night. *J Allergy Clin Immunol. 1999; 103:66-71.*

18. Bettiol J, Sele J, Henket M, et al. Cytokine production from sputum cells after allergenic challenge in IgE-mediated asthma. *Allergy 2002; 57:1145 –1150.*

19. Liu LY, Swensen CA, Kelly EA, Kita H, Busse WW. The relationship of sputum eosinophilia and sputum cell generation of IL-5. *J Allergy Clin Immunol 2000; 106:104-109.*

20. Komai-Koma M, Mckay A, Thomson L, et al. Immuno-regulatory cytokines in asthma: IL-15 and IL-13 in induced sputum. *Clinl Exp Allergy 2001; 31:1441-1448.*

21. Hamzaoul A, Ben Brahim M, Zhioua A, Ayed Kh, Hamzaoul K. Inflammatory response in induced sputum mononuclear cells from patients with acute exacerbation of asthma. *Mediators Inflamm 2000; 9:147-153.*

22. Boniface S, Koscher K, Manassier E, et al. Assessment of T lymphocyte cytokine production in induced sputum from astmatics: a flow cytometry study. *Clin Exp Allergy 2003; 33:1738-1743.*

23. ACCP-ATS Joint Committee on Pulmonary Nomenclature. Pulmonary terms and symbols. *Chest 1975; 67:5833-5893.*

24. Paggiaro PL, Chanez P, Holz O, et al. Sputum induction. *Eur Respir J 2002; 20:3S-8S.*

25. Pizzichini E, Pizzichini MM, Leigh R, Djukanovic R, Sterk PJ. Safety of sputum induction. *Eur Respir J 2002 ;20:9S-18S.*

26. Griffiths-Johnson DA, Collins PD, Rossi AG, Jose PJ, Willians TJ. The chemokine, eotaxin, activates guinea-pig eosinophils *in vitr*o, and causes their accumulation into the lung *in vivo. Biochem Biophys Res Commun 1993; 197:1167–1172.*

27. Jose, PJ, Griffiths-Johnson DA, Collins PD, et al. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea-pig model of allergic airways inflammation. *J Exp Med 1994; 179:881–887.*

28. Mattoli S, Stacey MA, Sun G, Bellini A, Marini M. Eotaxin expression and eosinophilic inflammation in asthma. *Biochem Biophys Res Commun.1997; 236:299–301.*

29. Rankin SM, Conroy DM. Williams TJ. Eotaxin and eosinophil recruitment: implications for human disease. *Mol Med Today 2000; 6:20-27.*

30 Forssmann U, Uguccioni M, Loetscher P, et al. Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. *J Exp Med 1997; 185:2171–2176.*

31. White JR, Imburgia C, Dul E, et al. Cloning and functional characterization of a novel human CC chemokine that binds to the CCR3 receptor and activates human eosinophil. *J Leukoc Biol 1997; 62:667–675.*

32. Ying S, Robinson DS, Meng O, et al. CC chemokines in allergen-induced late-phase cutaneous re-sponses in atopic subjects: association of eotaxin with early 6-hour eosinophils, and of eotaxin-2 and monocyte chemoattractant protein-4 with the later 24-hour tissue eosinophilia, and relationship to basophils and other CC chemokines (Monocyte chemoattractant protein-3 and RANTES). *J Immunol 1999; 163:3976–3984.*

33. Jatakanon A, Kharitonov S, Lim S, Barnes PJ. Effect of differing doses of inhaled budesonide on markers of airway in inflammation in patients with mild asthma. *Thorax 1999; 54:108 –114.*

34. Leung DYM, De Castro M, Szefler SJ, Chrousos GP. Mechanisms of glucocorticoid-resistant asthma. *Ann N Y Acad Sci 1998; 840:735-746.*

35. Walsh SD, Grant IWB. Corticosteroids in the treatment of asthma. *BMJ 1966; 2:796-807.*

36. Mukaida N, Morita M, Ishikawa Y, et al. Novel mechanisms of glucocorticoid-mediated gene repression: nuclear factor-Kappa B is target for glucocorticoid-mediated interleukin-8 gene expression. *J Biol Chem 1994; 269:3289-3295.*

37. Kelley J. Cytokines in the lung. *Am Rev Respir Dis 1990; 141:765-788.*

38. Hadjicharalambous C, Dent G, May RD, et al. Measurement of eotaxin (CCL11) in induced sputum supernatants: validation and detection in asthma. *J Allergy Clin Immunol 2004; 113:657-662.*

Table 1: Characteristics of the subjects studied

	Control	Asthma IC	Asthma OC	
			Pre-treatment	Post-treatment
n	21	21	09	
Sex (M/F)	6/15	7/14	6/3	
Age (years) ± SD	41.1 ± 12.9	48.3 ± 10.5	30.0 ± 13.1	
FEV_1 (%) ± SD	95.6 ± 10.6*	70.8 ± 13.1	69.4 ± 7.7	74.1 ± 4.7
Medication in use (% of patients)				
Inhaled corticosteroid	-----	100%	-----	-----
Bronchodilator	-----	100%	100%	100%
Oral corticosteroid	-----	-----	-----	100%
Sputum weight (g) ± SD	6.2 ± 3.3	7.6 ± 3.3	6.3 ± 3.9	6.4 ± 4.9
Total cell count (x 10^6) ± SD	3.3 ± 3.1	6.3 ± 3.2*	4.0 ± 3.2	2.5 ± 0.8
Duration of induction (min)	19.7 ± 1.1	18.5 ± 2.3	13.8 ± 4.1*	13.8 ± 4.1*

FEV_1: forced expiratory volume in the first second. Asthma IC: asthmatics treated with inhaled corticosteroid. Asthma OC: asthmatics treated with oral corticosteroids during 14 days.

* $p < 0.05$ compared to the other two groups.

Table 2 - Production of cytokines by LPS-stimulated and non-stimulated adherent cells and PHA-stimulated and non-stimulated non-adherent cells from induced sputum in the asthma IC group.

	Non-stimulated	**Stimulated**
Adherent cells		
TNF-α (pg/ml)	555 ± 833	816 ± 1538
IL-8 (pg/ml)	864 ± 89	826 ± 35
IL-1β (pg/ml)	100 ± 105	89 ± 94
Non-adherent cells		
Eotaxin 2 (pg/ml)	122 ± 119	115 ± 101
TNF-α (pg/ml)	3637 ± 2834	3763 ± 3364

Table 3 - Production of cytokines by adherent cells and non-adherent cells from induced sputum of the control and asthma IC groups.

	Control group	**Asthma IC Group**
Adherent cells		
TNF-α (pg/ml)	1817 ± 901	833 ± 571
IL-8 (pg/ml)	889 ± 13	901 ± 72
IL-1β (pg/ml)	91 ± 60	94 ± 62
Non-adherent cells		
Eotaxin 2 (pg/ml)	36 ± 41	131 ± 108*
TNF-α (pg/ml)	1678 ± 1976	3778 ± 276

*$p<0.05$ compared to control group.

Table 4 – Differential cell counts in sputum and sputum cell culture

Eosinophils (%)	**Groups**			
	Control	Asthma IC	Asthma OC pre-treatment	Asthma OC post-treatment
Sputum	1.8 ± 3.2	25.7 ± 31.2*	11.2 ± 12.1*	0.8 ± 1.4
Adherent cells	0.2 ± 0.5	8.7 ± 11.8	9.2 ± 6.8^{+}	0.9 ± 1.4
Non-adherent cells	0	23.8 ± 32.9^{+}	17.1 ± 6.3^{+}	1.6 ± 1.8
Lymphocytes (%)				
Sputum	16.1 ± 13.0	4.5 ± 1.5^{+}	8.6 ± 3.7	5.8 ± 2.5
Adherent cells	19.8 ± 7.7	9.8 ± 1.7	31.0 ± 26.1	1.6 ± 2.2
Non-adherent cells	3.6 ± 4.2	6.2 ± 7.3	17.6 ± 15.6^{+}	7.0 ± 6.5
Neutrophils (%)				
Sputum	38.4 ± 21.6	22.3 ± 9.2**	54.1 ± 20.6	66.9 ± 9.2^{+}
Adherent cells	0.5 ± 1.0	0.8 ± 1.3	25.0 ± 23.1^{+}	3.2 ± 4.5
Non-adherent cells	0.6 ± 1.2	0	33.6 ± 28.3	2.0 ± 0.2
Macrophages (%)				
Sputum	43.6 ± 22.9	53.6 ± 23.9$^{\#}$	34.7 ± 17.3	26.5 ± 20.2
Adherent cells	79.8 ± 9.1	80.4 ± 12.1	40.1 ± 14.6^{+}	94.0 ± 6.1
Non-adherent cells	91.4 ± 26.4	70.0 ± 38.1	31.6 ± 7.4	88.6 ± 7.9

- $p < 0.05$ compared to asthma OC post-treatment and control group; ** $p < 0.05$ compared to asthma OC pre- and post-treatment; $^{+}$ $p < 0.05$ compared to control group; $^{\#}$ $p < 0.05$ compared to asthma OC post-treatment.

Figure Legends

Figure 1 - Viability of sputum cells in culture. Cells were obtained from 21 asthmatic patients (asthma inhaled corticosteroid group) by sputum induction. * p = 0.004 in comparison with previous time-points for both adherent and non-adherent cells.

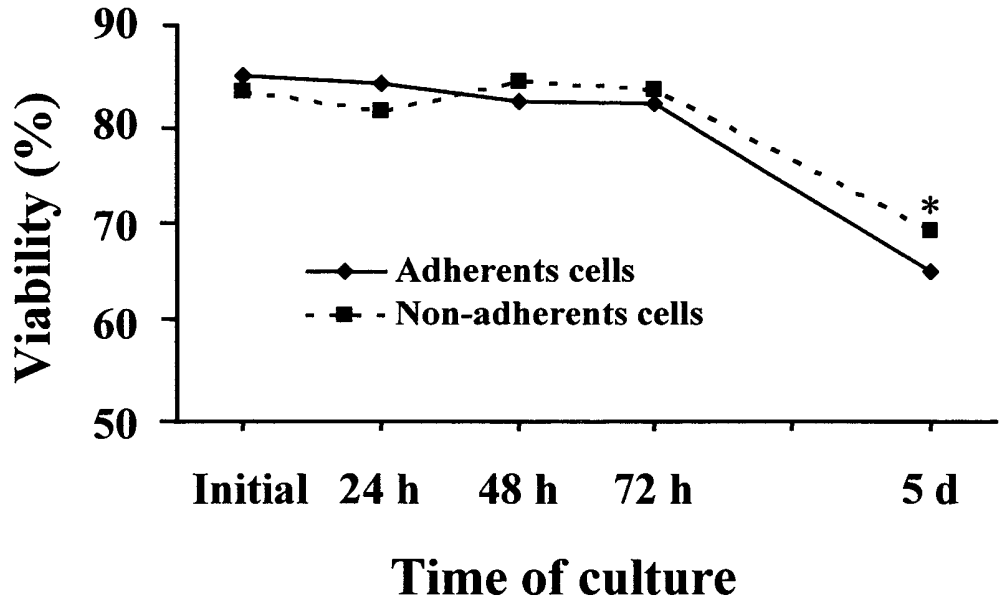

Figure 2 - Production of eotaxin 2 by sputum cell culture (non-adherent cells) of normal subjects (controls), asthmatics treated with an inhaled corticosteroid (IC), and asthmatics treated with an oral corticosteroid (OC). * $p < 0.05$ compared to both control and asthma OC groups. The horizontal bars indicate means of values.

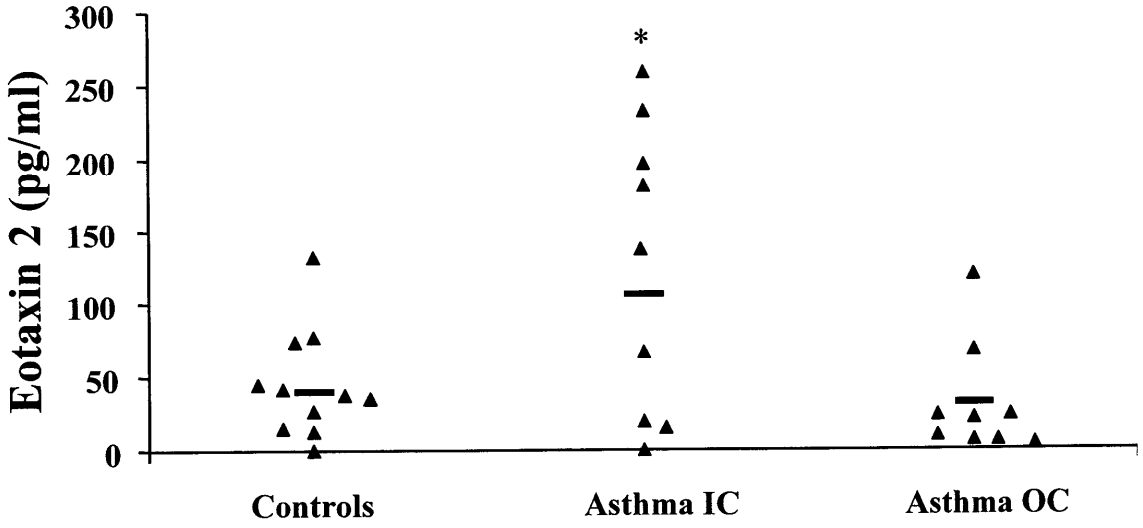

Figure 3 - Response to corticosteroid treatment was measured as change (Δ) in production of eotaxin 2 (Δ = pre-treatment – post-treatment) tested in both induced sputum and sputum cell culture (non-adherent cells). Asthmatics were treated with oral prednisone 40 mg/d for 14 days. The horizontal bars indicate means of values.