

Cytokine mRNA expression in asthma is not restricted to the large airways

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Background: Although previous studies have established the presence of an eosinophil-rich cellular infiltrate in the small airways of asthmatic lungs, the expression of cytokines within the peripheral airways has been largely unexplored. The purpose of our study was to test the hypothesis that T_{H2}-type cytokines are increased in the peripheral airways and parenchyma of asthmatic lungs.

Methods: The presence of messenger ribonucleic acid (mRNA) encoding both T-helper (T_{H1})-type (IL-2, interferon- γ) and T_{H2}-type (IL-4, IL-5) cytokines in surgically resected lungs from six asthmatic and 10 nonasthmatic subjects was determined by in situ hybridization. Colocalization of IL-5 mRNA within the large and small airways was performed by simultaneous in situ hybridization and immunocytochemistry.

Results: Expression of IL-5 mRNA-positive cells was significantly increased in the large and small airways and in the lung parenchyma of asthmatic subjects compared with nonasthmatic subjects. In the asthmatic individuals, the expression of IL-5 mRNA was increased in the small airways compared with the large airways. There was also an increase in the number of cells expressing IL-4 mRNA in the large and small asthmatic airways compared with the nonasthmatic airways. In contrast, the numbers of IL-2 and interferon- γ mRNA-positive cells did not differ between asthmatic and nonasthmatic individuals.

Conclusions: We conclude that there is an increased expression of T_{H2}-type cytokines within the peripheral airways of asthmatic lungs and suggest that the small airways contribute to the pathophysiology of asthma. (*J Allergy Clin Immunol* 1998;101:386-90.)

Key words: Cytokines, asthma, inflammation, lung resection, lymphocytes

The introduction of fiberoptic bronchoscopy has substantially advanced our understanding of the pathogenesis of bronchial asthma, particularly with respect to the central airways that can be reached with this method.^{1,2} Recently, two studies based on resected³ and postmortem lungs⁴ demonstrated a significant inflammatory

Abbreviations used

BAL:	Bronchoalveolar lavage
cDNA:	Complementary deoxyribonucleic acid
cRNA:	Complementary RNA
IFN- γ :	Interferon- γ
mRNA:	Messenger RNA
Pi:	Internal perimeter
RNA:	Ribonucleic acid
T _{H1} , T _{H2} :	T-helper cells

component in the wall and lumen of the small airways and in the lung parenchyma. The presence of such an inflammatory response contributes to both the structural changes observed in asthmatic airways and the airway obstruction that characterizes asthma.^{5,6} Cytokines are thought to be central mediators in the regulation of inflammatory responses, and bronchial biopsy specimens and bronchoalveolar lavage (BAL) fluid from asthmatic subjects characteristically display a preponderance of T-helper (T_{H2})-type cytokines, namely IL-4 and IL-5.⁷⁻⁹ Produced locally within the airways, these may act to promote IgE synthesis¹⁰ as well as enhancing eosinophil recruitment and survival within the tissue.¹¹⁻¹³ Although previous studies have demonstrated the presence of inflammation throughout the whole length of the airways in asthmatic individuals,^{3,4,14} there have been no studies to date examining cytokine messenger ribonucleic acid (mRNA) expression in large and small airways and parenchyma from asthmatic individuals. The aim of this study was to use in situ hybridization to examine the expression of T_{H1}-type (IL-2, interferon- γ [IFN- γ]) and T_{H2}-type (IL-4, IL-5) cytokines in the small airways and parenchyma of resected lung specimens from patients with asthma and nonasthmatic control subjects. The data collected were then used to test the hypothesis that the expression of T_{H2}-type cytokines, in particular IL-5, would be increased in the large and small airways of asthmatic subjects compared with nonasthmatic control subjects.

METHODS

Subjects

Lung tissue was obtained from 16 subjects selected from a cohort of 108 individuals entering the St. Paul's Hospital Lung Study between 1991 and 1995. The clinical characteristics of these asthmatic and nonasthmatic patients have been described previously.³ Briefly, patients undergoing lung resection for bronchial carcinoma who fulfilled the clinical criteria used to

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Supported by the Respiratory Health Network Center of Excellence. Dr. Minshall is a recipient of a Medical Research Council/Canadian Lung Association Fellowship.

Received for publication July 29, 1997; revised Oct. 24, 1997; accepted for publication Oct. 28, 1997.

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0091-6749/98 \$5.00 + 0 1/1/87175

establish a diagnosis of asthma ($n = 6$) were matched with 10 individuals who did not demonstrate these features. Diagnostic criteria included prior physician diagnosis and treatment for asthma, documented evidence of variable airflow limitation greater than 15%, and airway hyperresponsiveness (<16 mg/ml methacholine). Three of these asthmatic patients had received inhaled steroid treatment for their asthma, although only two were taking steroids at the time of resection. The study was approved by the St. Paul's Hospital Ethical Committee, and informed consent was obtained from all subjects.

Tissue preparation

The preparation of the lung specimens for in situ hybridization has been described previously.³ Briefly, the lung tissue was allowed to collapse after resection, and the tumor was sampled for histologic evaluation. The pleural surface was then sutured shut, and the lung was inflated through bronchi with optimal cutting temperature media diluted 1:1 in normal saline. The inflated lung was then frozen over liquid nitrogen, and cores of lung tissue (1.5×2 cm) were taken with the use of a power-driven drill and hollow bit. The frozen cores of lung tissue were then stored at -70°C until subsequent use.

Probe preparation

Radiolabeled complementary ribonucleic acid (cRNA) probes coding for IL-2, IL-4, IL-5, and IFN- γ mRNA were prepared from complementary deoxyribonucleic acid (cDNA) as described previously.⁷ In brief, cDNA was inserted into PGEM vectors, linearized, and transcribed in vitro in the presence of ^{35}S -UTP and either SP6 or T7 polymerases. Antisense (complementary to mRNA) and sense probes (identical to mRNA) were prepared.

In situ hybridization

Twenty-five cores of frozen lung tissue were selected from the 16 study subjects (eight cores of tissue from six patients with asthma and 17 from the nonasthmatic subjects) and shipped to the Meakins-Christie Laboratories with a code to present the tissue in a random fashion. From these cores of tissue, $10\text{ }\mu\text{m}$ cryostat tissue sections were cut, fixed in 4% paraformaldehyde for 30 minutes, washed twice in 15% phosphate-buffered sucrose, and dried overnight at 37°C . Sections of lung tissue were then processed for in situ hybridization for IL-2, IL-4, IL-5, and IFN- γ mRNA as previously described.^{7,15} Briefly, the tissue sections were permeabilized with Triton X-100 and proteinase K solution ($1\text{ }\mu\text{g/ml}$) in 0.1 mol/L Tris containing 50 mmol/L for 20 minutes at 37°C . The samples were subsequently incubated with 0.1 mol/L triethanolamine and 0.5% acetic anhydride for 20 minutes at 37°C to prevent nonspecific binding of the ^{35}S -labeled cRNA probes. Prehybridization of the samples was carried out in 50% formamide in $2 \times$ standard saline citrate for 15 minutes at 37°C . Hybridization was carried out with a hybridization mixture containing the appropriate sense or antisense probes (0.75×10^6 cpm/slide). Posthybridization involved high stringency washing of the samples in decreasing concentrations of standard saline citrate at 42°C . To remove any unbound RNA probes, the samples were washed with RNase solution for 20 minutes at 42°C . The samples were then dehydrated with increasing concentrations of ethanol and were then air dried. After this, the samples were dipped in Amersham LM-2 emulsion and then exposed for a period of 10 days. The autoradiographs were then developed in Kodak D-19 developer, fixed, and coun-

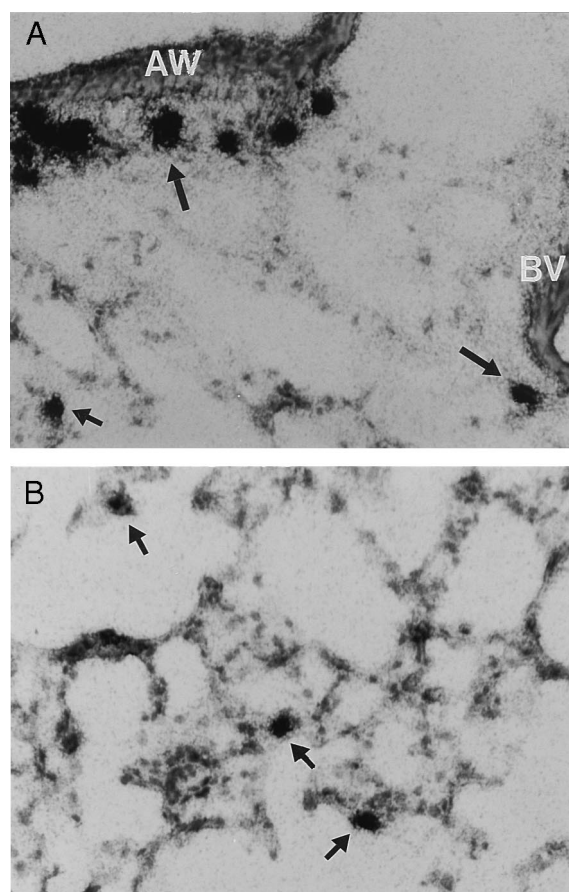


FIG. 1. Representative autoradiographs of in situ hybridization of resected lungs from patients with asthma, with ^{35}S -labeled antisense cRNA probes coding for IL-5 mRNA-positive cells. The positive hybridization signals are dense collections of silver grains overlying the cells. **A**, IL-5 mRNA-positive cells can be seen in close proximity to small airways (AW) and blood vessels (BV). **B**, These cells are also to be found within the lung parenchyma.

terstained with hematoxylin. The samples were then mounted with a coverslip and examined under a graduated microscope for positive signals.

Combined immunocytochemistry and in situ hybridization

To ascertain whether the increased expression of IL-5 mRNA within the small airways was attributed to either T cells or eosinophils, we simultaneously applied radiolabeled in situ hybridization with immunocytochemistry for T cells (CD3+) and eosinophils (MBP) as previously described in detail elsewhere.⁹ Briefly, sections ($5\text{ }\mu\text{m}$) of frozen lung tissue were cut from two asthmatic individuals and underwent immunostaining with monoclonal anti-CD3 (Becton-Dickenson) and anti-MBP (BMK-13; a gift from Dr. R. Moqbel, University of Alberta, Canada) antibodies. After visualization of the positive immunostaining with the alkaline phosphatase anti-alkaline phosphatase technique, the sections underwent a modified in situ hybridization for IL-5 mRNA.

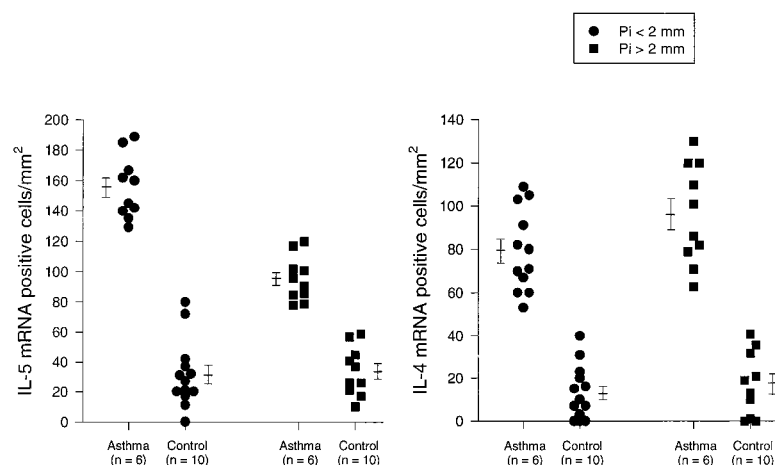


FIG. 2. Expression of IL-4 and IL-5 mRNA in small (< 2 mm Pi) and large (> 2 mm Pi) airways of asthmatic subjects and nonasthmatic control subjects. For airways less than 2 mm Pi, values given are means of 10 airways from four patients with asthma and 14 airways from 10 nonasthmatic control subjects. For airways greater than 2 mm Pi, values given are means of 10 airways from six patients with asthma and 10 airways from 10 nonasthmatic control subjects.

Quantification of cytokine mRNA-positive cells in the airway wall

The quantification of cytokine mRNA-positive cells was determined as previously outlined.³ Cells positive for cytokine mRNA expression present within the airway wall were counted blindly and expressed as the number of positive cells per square millimeter of the airway wall. For a quantitative comparison, airways were divided into those less than 2 mm and those greater than 2 mm in their internal perimeter (Pi). To determine the number of cytokine mRNA-positive cells as a fraction of the alveolar tissue, a point-counting system was used, as previously described.¹⁶ Cells positive for IL-5 mRNA and either CD3 or MBP were quantified as a percentage of the total number of cells expressing IL-5 mRNA ($\pm$ SEM). When all the data were obtained, they were sent to the Pulmonary Research Laboratory, Vancouver, where the code was broken.

Statistics

Comparisons between cell counts in patients with asthma and nonasthmatic control subjects were performed with a parametric analysis of variance with a post hoc Bonferroni correction. Probability (p) values less than 0.05 were considered statistically significant.

RESULTS

The asthmatic and nonasthmatic patients were well matched for age, sex, and cigarette smoking history³ ($p > 0.05$). The subjects with asthma had a mean percent predicted FEV₁ and standard deviation of $82.5\% \pm 22.1\%$, which was similar to that of the nonasthmatic control subjects: $89.2\% \pm 15.3\%$ ($p > 0.05$). Histologic studies of the lungs revealed that the Pi of the airways varied between 0.8 and 16.4 mm, with a similar frequency of distribution of size between the two groups.³

Positive in situ hybridization signals for cytokine mRNA were observed as dense collections of silver grains overlying cells on autoradiographs only when the

antisense probes for IL-4, IL-5, IL-2, and IFN- γ were used. No signals were observed with the sense probes or from preparations pretreated with RNase. Using this technique, we observed IL-5 mRNA-positive cells situated in close proximity to airways, as well as near blood vessels and within the lung parenchyma (Fig. 1).

Cytokine mRNA-positive cells were enumerated in the different-sized airways, and the large (> 2 mm Pi) and small airways (< 2 mm Pi) from subjects with asthma displayed an increase in the number of IL-4 and IL-5 mRNA-positive cells compared with those from nonasthmatic individuals (Fig. 2; $p < 0.001$). In the asthmatic individuals, the expression of IL-5 but not IL-4 mRNA was increased in the small airways compared with the large airways (Fig. 2; $p < 0.001$). There were no differences in the numbers of IL-2 and IFN- γ mRNA-positive cells between asthmatic and nonasthmatic subjects in the large or small airways (Fig. 3; $p > 0.05$).

Using simultaneous in situ hybridization and immunocytochemistry on frozen lung tissue from two asthmatic individuals, 85% (± 3) of the IL-5 mRNA-positive cells were CD3 positive and only 6% (± 2) of the IL-5 mRNA-positive cells were MBP positive in small airways. In large airways, similar percentages were obtained for IL-5/CD3-positive cells ($81\% \pm 6\%$) and for IL-5/MBP-positive cells ($8\% \pm 3\%$).

Cytokine expression was also examined in the parenchyma of the asthmatic and nonasthmatic individuals. When expressed as a volume fraction of the alveolar wall tissue, the number of IL-5 mRNA-positive cells presented was greater in patients with asthma than in the nonasthmatic control subjects (Fig. 4; $p < 0.05$). However, there was no difference in the numbers of IL-4 mRNA-positive cells in the lung parenchyma of asthmatic subjects compared with nonasthmatic control subjects ($p > 0.05$).

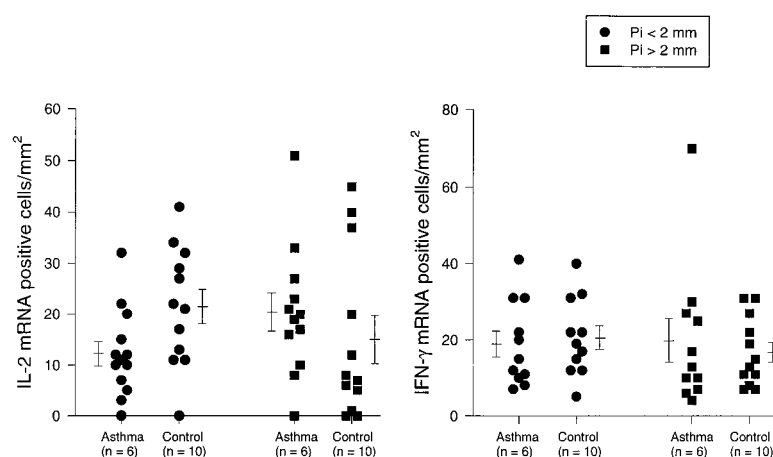


FIG. 3. Expression of IL-2 and IFN- γ mRNA in small (< 2 mm Pi) and large (> 2 mm Pi) airways of asthmatic subjects and nonasthmatic control subjects. For airways less than 2 mm Pi, values given are means of 12 airways from four patients with asthma and 11 airways from 10 nonasthmatic control subjects. For airways greater than 2 mm Pi, values given are means of 11 airways from six patients with asthma and 11 airways from 10 nonasthmatic control subjects.

DISCUSSION

This study demonstrates an increase in the numbers of IL-4 and IL-5 mRNA-positive cells within the airways of patients with asthma compared with nonasthmatic control subjects matched for age, sex, smoking history, and airway size. More importantly, cells expressing IL-5 mRNA were significantly increased in airways less than 2 mm Pi in the asthmatic subjects compared with the nonasthmatic control subjects, and in the asthmatic subjects these were found to be significantly elevated compared with the larger (> 2 mm Pi) airways. The presence of increased numbers of IL-4 and IL-5 cytokine mRNA-positive cells within the larger airways is consistent with previous bronchial biopsy and BAL findings of asthmatic airways.^{7-9, 17} The finding of increased numbers of IL-5 mRNA-positive cells within the smaller (< 2 mm Pi) airways of asthmatics, however, is novel and lends support to previous findings indicating that the inflammatory response in asthma is not restricted to the larger airways.^{3, 4}

The differences between our asthmatic and nonasthmatic groups were observed despite limitations imposed by the choice of subject criteria. These included the use of resected lungs from patients with lung carcinoma and the presence of cigarette-related inflammatory changes that were present to a similar extent in both groups.³ In addition, our asthmatic patients were relatively mildly affected, and thus the extent of lung inflammatory changes may be less obvious than in more severely affected individuals. Nevertheless our results extend results taken from biopsy specimens and BAL fluid in that T_H2-type cytokine expression is present within peripheral airways not routinely sampled during fiberoptic bronchoscopy.

Previous studies of airway physiology in asthmatic subjects and normal control subjects have indicated that the peripheral airways exert a major contribution to the

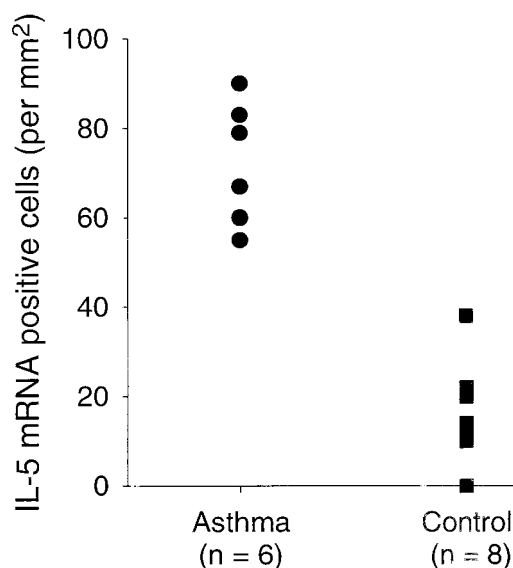


FIG. 4. Expression of IL-5 mRNA within the lung parenchyma of asthmatic subjects ($n = 6$) and nonasthmatic control subjects ($n = 8$). Values given are the means of the individual subjects.

overall increase in airway obstruction in subjects with asthma.⁵ Moreover, it has been predicted by quantitative histology that these airways are the site of increased airway responsiveness in asthma.⁶ Alterations in cytokine expression within the small (< 2 mm Pi) airways may contribute to the pathophysiology of asthma by coordinating the observed inflammatory infiltrate.³ Indeed, the enhanced expression of IL-5 mRNA within the smaller airways is consistent with our previous findings in these same asthmatic patients that demonstrated increased numbers of activated (EG2+) eosinophils in airways less than 2 mm Pi in comparison with airways greater than 2 mm Pi. IL-5 is central to many aspects of

eosinophil biology. In particular, it appears critical in the differentiation of eosinophil precursors and stimulates their maturation, activation, and recruitment into the tissue.^{10, 11} Furthermore, this cytokine prevents eosinophil apoptosis and may contribute to the longevity of these cells within the airways.¹³ Therefore the presence of this cytokine within the small airways may be expected to contribute to the eosinophilic inflammation.

The recognition of cytokine expression within the small airways is important not only in the pathophysiology of asthma but has diagnostic and therapeutic implications. Earlier biopsy studies have suggested that one of the mechanisms of action of inhaled steroids in attenuating allergic inflammation is to inhibit cytokine expression in the proximal airways, particularly IL-4 and IL-5.^{17, 18} Whether these agents are effective at inhibiting cytokine expression within the periphery of the lung remains to be established. If not, it may be suggested that the inflammatory component persists in individuals treated with inhaled steroids. Such inflammation could then result in airway fibrosis and irreversible changes in lung function. Indeed, long-term studies of inhaled steroids in asthmatic subjects suggest some component of airway obstruction, and hyperresponsiveness persists in these treated individuals.^{19, 20} Whether this explanation can account for the greater efficacy of oral steroids remains to be determined.

Previous data in bronchial biopsies have suggested that the majority of the cells expressing IL-4 and IL-5 mRNA in asthmatic airways are CD4⁺ T lymphocytes, with lesser contributions made by eosinophils and mast cells.⁹ Although the number of T cells within the airways is not related to airway size,³ the preponderance of IL-5 mRNA-positive cells could be attributed to the selective recruitment of IL-5 mRNA-expressing T cells or their switching to IL-5 mRNA expression within the small airways and lung parenchyma. Alternatively, within the smaller airways and lung tissue, eosinophils themselves or even mast cells may be a more important source of this cytokine.

In summary, these studies have demonstrated that a significantly increased expression of IL-4 and IL-5 mRNA is evident within the small (< 2 mm Pi) airways of asthmatic individuals. Numbers of cells expressing IL-2 and IFN- γ mRNA were not significantly elevated in these subjects. These results support previous reports of eosinophilic airway inflammation in the lung periphery and implicate the small airways in the pathophysiology of asthma.

The authors would like to thank Ms. Elsa Schotman and Ms. Zivart Yasruel for technical assistance.

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