

Upregulation of α GM-CSF-receptor in nonatopic asthma but not in atopic asthma

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Background: Intrinsic asthma is characterized by an increased number of activated eosinophils and macrophages and an increased expression of the hematopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) in the bronchial mucosa.

Objective: This study was carried out to investigate the expression of α GM-CSF receptor (α GM-CSFr) messenger RNA and protein in the bronchial mucosa of patients with intrinsic or atopic asthma and of control subjects and to correlate the expression of α GM-CSFr to the number of EG2⁺ cells (eosinophils) and CD68⁺ cells (macrophages) and pulmonary function.

Methods: Nineteen patients with stable asthma (9 with atopic and 10 with intrinsic asthma) and 22 normal control subjects (12 atopic and 10 nonatopic subjects) were recruited, and FEV₁ (percent predicted) and PC₂₀ were measured before bronchoscopy. Endobronchial biopsy specimens were obtained and examined for membrane-bound α GM-CSFr by using in situ hybridization and immunocytochemistry.

Results: α GM-CSFr mRNA- and protein-positive cells were identified in biopsy specimens from all four groups studied. There was no significant difference in the number of cells expressing α GM-CSFr mRNA and protein in patients with atopic asthma compared with atopic and nonatopic control subjects. However, the numbers of α GM-CSFr mRNA- and protein-positive cells were significantly higher in nonatopic patients with asthma compared with atopic patients with asthma and atopic and nonatopic control subjects ($p < 0.001$). In the patients with intrinsic asthma, the number of α GM-CSFr mRNA-positive cells per millimeter of basement membrane correlated with numbers of CD68⁺ cells ($r^2 = 0.87$, $p < 0.001$) but not with EG2⁺ cells, and colocalization studies demonstrated that 80% of the cells expressing α GMCSFr mRNA were CD68⁺. The expression of GM-CSF was also significantly increased in patients with intrinsic asthma compared with those with atopic asthma and control subjects ($p < 0.05$). In addition, in intrinsic asthma, there

was a correlation between α GM-CSFr mRNA and FEV₁ ($r^2 = 0.61$, $p < 0.05$).

Conclusion: These results demonstrate that elevated numbers of cells expressing α GM-CSFr can be detected in nonatopic asthma but not in atopic asthma and suggest that this increased expression is predominantly macrophage-associated and may play an important pathophysiologic role in intrinsic asthma. (J Allergy Clin Immunol 1997;99:666-72.)

Key words: Intrinsic asthma, macrophages, GM-CSF receptor

Asthma is characterized by episodic airway obstruction and increased bronchial responsiveness to the inhalation of nonspecific irritants.^{1,2} Numerous studies have now linked these features to chronic infiltration of the bronchial mucosa with activated eosinophils, T lymphocytes, and macrophages.³⁻⁷ The predominant cell types in the airway mucosa of normal and asthmatic subjects are lymphocytes and macrophages.^{3,4} In relative terms the increase in the numbers of eosinophils in airways of patients with asthma compared with those of control subjects is the greatest of any inflammatory cell type; however, the absolute number of eosinophils present still only accounts for less than 25% of the total inflammatory cell population in the bronchial mucosa of patients with asthma.⁴ In contrast, although there are smaller changes in the relative numbers of lymphocytes and macrophages in airways of patients with asthma compared with those of control subjects, the increases in the absolute numbers of these cells in asthmatic subjects is of the same order as that seen for the eosinophils.⁴ Furthermore, there is also a marked increase in the degree of activation of these two cell populations in airways of patients with asthma.^{5,7-11} Although all these inflammatory cells have been implicated in the pathophysiology of both atopic and intrinsic asthma,¹²⁻¹⁷ there is evidence to suggest that the macrophage may play a relatively more important role in intrinsic asthma.⁴

The hematopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) is an important cytokine for the growth and differentiation of eosinophils and macrophages in the bone marrow^{18,19} and may also be important in the activation of these cells at peripheral tissue sites.^{20,21} GM-CSF is upregulated in the bronchoalveolar lavage cells and biopsy specimens of patients with asthma compared with those from normal control subjects,^{22,23} and this upregulation is associated

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Supported by Network Centre of Excellence for Respiratory Diseases and Medical Research Council, Canada.

Received for publication Aug. 5, 1996; revised Oct. 22, 1996; accepted for publication Nov. 25, 1996.

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0091-6749/97 \$5.00 + 0 1/1/79715

Abbreviations used

α GM-CSFr:	α Subunit GM-CSF receptor
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
MBP:	Major basic protein

with increasing airflow limitation, bronchial hyperreactivity, and asthma score.²² In addition, GM-CSF has been shown to increase in the lungs²¹ and nasal mucosa after allergen challenge²⁴ and is decreased in activated CD4⁺ T lymphocytes in the blood after oral glucocorticoid therapy.²⁰ Although GM-CSF is upregulated in the bronchial mucosa of patients with atopic or intrinsic asthma, there is evidence that it may play a relatively more important role in intrinsic asthma.^{25, 26}

The human GM-CSF receptor (GM-CSFr) has been identified *in vitro* on various cells, including eosinophils and monocytes.²⁷⁻³⁰ The GM-CSFr is a heterodimer consisting of two polypeptide chains, α and β ,³¹ both of which belong to the cytokine receptor superfamily.³² The β subunit is shared with the receptors for human IL-3 and human IL-5.^{30, 33, 34} However, the α chain is specific for binding of human GM-CSF.³⁵

In vivo, GM-CSF can be produced by T lymphocytes, macrophages, and eosinophils,^{7, 24} as well as by various structural cells such as epithelium³⁶ and stromal tissue.³⁷ The examination of GM-CSF receptor expression in the bronchial mucosa of patients with asthma would help determine the target cells for the increased amounts of GM-CSF that are present in patients with atopic or intrinsic asthma and would allow us to establish whether the predominant effect of GM-CSF in intrinsic asthma is on macrophages.

We hypothesize that there is an increase in local production of α GM-CSFr in airways of patients with asthma, that this occurs predominantly in intrinsic asthma, and that macrophages are a major site of α GM-CSFr expression. The specific objectives of our study were to investigate the expression of α GM-CSFr messenger RNA and protein in biopsy specimens of airways from atopic and nonatopic subjects with asthma compared with expression in atopic and nonatopic control subjects, to characterize the phenotype of the cells that express α GM-CSFr, and to examine the correlation between α GM-CSFr and the severity of asthma as measured by FEV₁ and airway hyperresponsiveness to methacholine. This was achieved by examining endobronchial biopsy specimens by using the techniques of *in situ* hybridization with a specific complementary RNA probe for α GM-CSFr, immunocytochemistry with a specific monoclonal antibody for α GM-CSFr, and simultaneous *in situ* hybridization and immunocytochemistry to determine the phenotype of the cells that expressed α GM-CSFr mRNA.

METHODS

Subjects

Bronchial biopsy specimens from 19 patients with stable asthma (9 with atopic asthma; and 10 with intrinsic asthma) and 22 control subjects (12 atopic and 10 nonatopic subjects) were obtained. The patients studied were recruited from the Allergy Clinic at the Royal Brompton National Heart and Lung Hospital, London, U.K., and the Department of Pneumology II, Hochgebirgsklinik, Davos Wolfgang, Switzerland. The diagnoses of asthma and atopy were as previously described.¹³ In brief, all the patients had typical clinical symptoms, documented reversibility of airflow obstruction, and increased airway responsiveness to inhaled aerosols of methacholine (PC₂₀ <8 mg/ml). None of the asthmatic patients had taken inhaled or oral corticosteroids or other prophylactic treatment in the preceding 3 months. The patients with atopic asthma had positive skin test responses to common inhalant aeroallergens. The atopic control subjects came from the Royal Brompton Hospital (patients with rhinitis were recruited from the allergy clinic or in response to hospital or newspaper advertisements), whereas the nonatopic healthy control subjects were all recruited from the local population. All subjects (asthmatic and control) were nonsmokers. The study was performed with written informed consent and with the approval of the Royal Brompton Hospital Ethics Committee.

Bronchial biopsies

The technique of fiberoptic bronchoscopy with bronchial biopsy specimens from the subsegmental airways in asthmatic and normal volunteers has been previously described.⁵ For each subject, baseline spirometry (FEV₁) was recorded, and a methacholine inhalation test was performed. Nebulized salbutamol was given to all subjects (asthmatic and control) before the bronchoscopy procedure. The biopsy specimens were immediately fixed in 4% paraformaldehyde, washed, frozen in isopentane cooled in liquid nitrogen, and stored at -70°C. They were subsequently shipped in dry ice to the Meakins-Christie Laboratories, McGill University (Montreal, Quebec, Canada) for processing for *in situ* hybridization.

In situ hybridization

Human α GM-CSFr complementary DNA was used as a template for synthesizing an RNA probe coding for α GM-CSFr.^{33, 38} The α GM-CSFr riboprobe contained the sequence complementary to the exon coding for the transmembrane region of the α GM-CSFr, thus ensuring that this riboprobe was specific for the membrane-anchored isoform of this receptor.³⁸ The DNA template was inserted into a pGEM vector and linearized with appropriate enzymes. Radiolabeled anti-sense (or sense) transcripts were synthesized, and *in situ* hybridization was performed on 10 μ m cryostat sections from the biopsy specimens as previously described by our group.³⁹⁻⁴¹ *In situ* hybridization for GM-CSF messenger RNA was also performed by using the above protocols.

Immunocytochemistry and simultaneous *in situ* hybridization and immunostaining

Sections from the control subjects (6 atopic and 6 nonatopic) and the asthmatic subjects (7 with atopic and 7 with intrinsic asthma), which were suitable to be processed for immunocytochemistry, were immunostained with a monoclonal antibody for the α subunit of the GM-CSF receptor (Pharmingen, San Diego, Calif.) by using the modified alkaline phosphatase-antialkaline phosphatase technique. Sections were also immuno-

stained with either a monoclonal antibody that recognizes activated eosinophils (anti-EG2) or a monoclonal antibody that recognizes macrophages (anti-CD68). For the simultaneous *in situ* hybridization and immunostaining, four separate, randomly selected sections from both the atopic and intrinsic asthma patient groups were tested. These were first immunostained with the alkaline phosphatase–anti-alkaline phosphatase technique with either the monoclonal antibody for CD68 or a monoclonal antibody that recognizes major basic protein (MBP) (BMK-13) and then processed for α GM-CSFr mRNA *in situ* hybridization by using a digoxigenin cRNA probe as previously described.⁴²

Quantification

Cells positive for α GM-CSFr mRNA and protein and for GM-CSF mRNA were counted in a 115 μ m zone along the entire length of the epithelial basement membrane. Cell counts were expressed per unit length (1 mm) of basement membrane. The numbers of cells coexpressing α GM-CSFr mRNA and MBP or CD68 are presented as a percentage of total cells expressing α GM-CSFr mRNA.

Statistics

Comparisons between cell counts in patients with asthma and normal control subjects were performed by using the Kruskal-Wallis analysis of variance for nonparametric data and the Mann-Whitney test for unpaired samples. Correlations were obtained by using the Pearson correlation coefficient. All tests used Bonferroni corrections for multiple comparisons. The results of the colocalization studies are presented as median ($\pm$ SEM) percentage values. Probability (*p*) values less than 0.05 were considered statistically significant.

RESULTS

Nineteen patients with stable asthma (9 with atopic and 10 with intrinsic asthma) and 22 control subjects (12 atopics and 10 nonatopic subjects) were studied. The median ages of the four groups of subjects were as follows: nonatopic control subjects, 22 years (range, 19 to 39 years); atopic control subjects, 24.5 years (range, 21 to 42 years); patients with atopic asthma, 26 years (range, 23 to 42 years) and; patients with nonatopic asthma, 53.5 years (range, 39 to 62 years). The asthmatic patients studied had relatively mild disease, and the severity of airflow obstruction and nonspecific bronchial hyperresponsiveness was similar in both the atopic and nonatopic asthma groups, with the median FEV₁ (percent predicted) levels of 85% (56% to 104%) and 75.5% (58% to 96%) and median methacholine PC₂₀ levels of 1.63 mg/ml (0.02 to 3.66 mg/ml) and 0.75 mg/ml (0.12 to 6 mg/ml), respectively.

There was no significant difference in α GM-CSFr mRNA or protein expression in patients with atopic asthma compared with atopic and nonatopic control subjects. However, the numbers of α GM-CSFr mRNA-positive cells per millimeter of basement membrane were significantly higher in the group with nonatopic asthma compared with the group with atopic asthma and atopic and nonatopic control subjects (*p* < 0.001) (Fig. 1, *a*). The numbers of α GM-CSFr protein-positive cells were also significantly higher in the patients with intrinsic asthma compared with patients with atopic asthma

and control subjects (*p* < 0.001) (Fig. 1, *b*). Although in the patients with intrinsic asthma, the numbers of α GM-CSFr mRNA-positive cells and α GM-CSFr protein-positive cells were similar for the group, there was no significant correlation between the two values in the same individual.

The number of macrophages (CD68⁺ cells) was significantly increased in the subjects with intrinsic asthma compared with the other groups (mean $\pm$ SEM values: atopic control subjects, 16.5 $\pm$ 3.2; nonatopic control subjects, 17.8 $\pm$ 2.2; patients with atopic asthma, 19 $\pm$ 4.1; and patients with intrinsic asthma, 45.6 $\pm$ 6.3; *p* < 0.005). The number of α GM-CSFr mRNA-positive cells per millimeter of basement membrane correlated with the number of CD68⁺ cells per millimeter of basement membrane both in the patients with intrinsic asthma (*r*² = 0.87, *p* < 0.001) (Fig. 1, *c*) and in patients with asthma as a group (*r*² = 0.74, *p* < 0.001), but in neither group was there a correlation with EG2⁺ cells.

In the patients with intrinsic asthma, simultaneous hybridization of the α GM-CSFr probe and immunostaining with anti-CD68 antibody showed that 80% $\pm$ 3.3% of the cells that were α GM-CSFr mRNA-positive were also positive for CD68, whereas 38% $\pm$ 5% of the CD68⁺ cells were positive for the mRNA of α GM-CSFr. Colocalization studies with MBP revealed that 15% $\pm$ 3% of the cells that were α GM-CSFr mRNA-positive were also positive for MBP. In the atopic asthma group, 75% $\pm$ 4% of the cells that were α GM-CSFr mRNA-positive were positive for CD68, and 6% $\pm$ 4% of the cells that were α GM-CSFr mRNA-positive were positive for MBP.

Analysis of the GM-CSF expression in the four groups of subjects studied demonstrated that there is a significant increase in the mean number of cells positive for GM-CSF in intrinsic asthma (median, 15.6; range, 3.9–40.9) compared with atopic asthma (median, 2; range, 0 to 37) and atopic (median, 0.4; range, 0 to 1.9) or nonatopic (median, 0.5; range, 0 to 5.6) control subjects (*p* < 0.05).

In addition, in the nonatopic asthmatic subjects, the number of positive cells expressing mRNA for α GM-CSFr correlated inversely with FEV₁ levels (*r*² = 0.61, *p* < 0.05) (Fig. 1, *d*). There were no significant correlations between α GM-CSFr and methacholine PC₂₀ values.

DISCUSSION

This study provides the first conclusive evidence of the upregulation of α GM-CSFr in the bronchial biopsy specimens of asthmatic subjects compared with those from control subjects. The results demonstrate that there are elevated numbers of α GM-CSFr mRNA- and protein-positive cells in nonatopic asthma but not in atopic asthma. We have also shown that the increased expression for α GM-CSFr is associated with an increased expression of GM-CSF and that the majority of α GM-CSFr-positive cells are macrophages. This is consistent with observations that GM-CSF is upregulated in

GM-CSF receptor

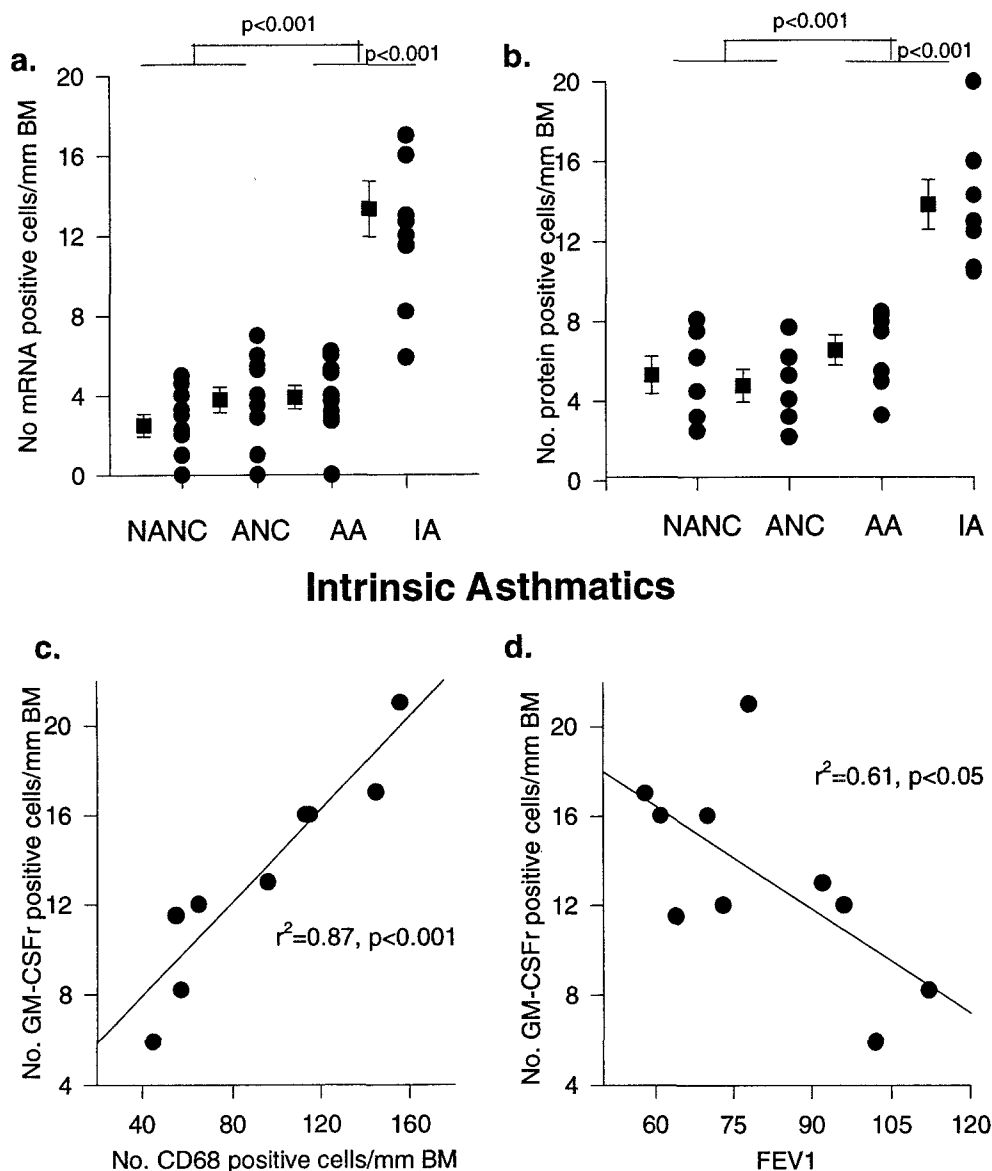


FIG. 1. **a**, Number of cells positive for α GM-CSFr mRNA in patients with atopic and nonatopic asthma compared with atopic and nonatopic control subjects, respectively. **b**, Number of positive cells for α GM-CSFr protein in patients with atopic and nonatopic asthma compared with atopic and nonatopic control subjects, respectively. **c**, Direct correlation between α GM-CSFr mRNA-positive cells and CD68⁺ cells in subjects with intrinsic asthma. **d**, Inverse correlation between α GM-CSFr mRNA-positive cells and FEV₁ in subjects with intrinsic asthma. NANC, Nonatopic normal control subjects; AC, atopic normal control subjects; AA, patients with atopic asthma; IA, patients with intrinsic asthma; BM, basement membrane. Results are expressed as either mean $\pm$ SEM values or as a Pearson's correlation coefficient.

the bronchial biopsy specimens of patients with intrinsic asthma,²⁵ and that there appears to be a recruitment of activated macrophages into the airways of patients with newly diagnosed and mild asthma in general⁴³ in subjects with intrinsic asthma in particular.²⁶ Although previous clinical studies have also suggested that macrophages may play a role in atopic asthma,^{44, 45} these studies

involved antigen challenge rather than subjects with relatively mild, stable asthma.

The α GM-CSFr has been identified on many cell lines including macrophages and eosinophils in vitro²⁵⁻²⁷ and in the skin⁴⁶; however, it has never previously been demonstrated within the bronchial mucosa. Our data provide evidence for the cellular localization of α GM-

CSFr, mainly to macrophages. We have shown that the number of macrophages present in patients with intrinsic asthma is greater than that in the other groups. This is in agreement with previous reports.⁴ It appears that the increased number of α GM-CSFr mRNA-positive macrophages in intrinsic asthma is predominantly due to increased numbers of macrophages present, although some of this increase is also due to an increase in the proportion of macrophages that express α GM-CSFr. In any case, our data indicate that there is an increase in the absolute numbers of α GM-CSFr mRNA-positive macrophages in intrinsic asthma, and this, combined with the evidence for increased expression of GM-CSF in intrinsic asthma, suggests that GM-CSF may play an important local role in stimulating macrophages in this group of patients.

Our results support the concept that there may be restricted lineage specificity of GM-CSF in the bronchial mucosa compared with that seen *in vitro*^{25, 27} because of the restricted expression of α GM-CSFr. Nevertheless, the results of *in vitro* studies and the finding that $15\% \pm 3\%$ of the α GM-CSFr mRNA-positive cells were MBP-positive in the patients with intrinsic asthma suggest that the numbers of eosinophils or basophils expressing α GM-CSFr *in vivo* could be potentially greater, which may be important in more severe asthma. Although MBP was used in the colocalization studies as a more sensitive marker of the eosinophil cell lineage than EG2, 5% of the α GM-CSFr mRNA-positive cells in the group with intrinsic asthma were negative for both CD68 and MBP. These α GM-CSFr mRNA-positive, CD68⁺, and MBP⁺ cells could be mast cells, lymphocytes, or fibroblasts. We did not perform any colocalization studies with markers for these cells.

The atopic and nonatopic asthmatic patients were matched for asthma severity. Hence, the increased expression of α GM-CSFr predominantly on macrophages in the group with nonatopic asthma suggests that, for a given level of asthma severity, macrophage activation may be more important in this group of patients. The relatively mild nature of the disease in the patients studied makes it important to further examine α GM-CSFr expression in more severe disease states to assess whether the increased number of α GM-CSFr-positive cells remains restricted to nonatopic asthma and macrophages or whether it can also be associated with increased eosinophil activation and more severe atopic asthma. It is also important to examine the response of α GM-CSFr expression to antigen challenge and steroid treatment.

The strong inverse correlation of macrophage-associated α GM-CSFr expression with FEV₁ in the tissue from patients with nonatopic asthma suggests that differential increases in macrophage numbers and activation status may be important in asthma. Although GM-CSF has been shown to inhibit apoptotic cell death of eosinophils and monocytes *in vivo*,⁴⁷ our results suggest that the latter may be relatively more important in the bronchial mucosa of patients with mild, stable intrinsic

asthma. However, activated eosinophils and macrophages can potentially interact with each other either directly, through eosinophil-derived GM-CSF,^{21, 48} or indirectly, through macrophage-derived proinflammatory cytokines that can increase T-lymphocyte activation, and hence, eosinophil activation.⁴⁹ Nevertheless, it is clear that the clinical presentation of intrinsic asthma is different from that of atopic asthma (differences in age and sex ratio), suggesting that there are at least some mechanistic differences between the two conditions and that different etiologic factors may be important in the establishment or progression of intrinsic asthma (e.g., recurrent exposure to certain environmental agents such as viruses, irritants, etc., in predisposed individuals⁵⁰).

Macrophage activation can be implicated in the pathogenesis of asthma through various mechanisms. Macrophages are antigen-presenting cells that have the capacity to upregulate HLA class I and class II expression and produce proinflammatory cytokines, thereby allowing amplification of chronic immune-mediated inflammatory responses. It has also been demonstrated that alveolar macrophages of patients with asthma have an increased secretory capacity and that they produce increased levels of eicosanoid mediators and superoxide anion,^{9, 10} all of which may have a direct effect on mucosal inflammation and bronchoconstriction in asthma.

In general, unlike FEV₁, methacholine PC₂₀ is considered a relatively poor measure of asthma severity. There was no correlation between α GM-CSFr expression and methacholine PC₂₀ in the subjects with intrinsic asthma. This suggests that the mechanisms underlying bronchial hyperreactivity differ from those that determine FEV₁. However, our results do not exclude the possibility of a specific GM-CSFr-positive macrophage phenotype being associated with bronchial hyperreactivity, because macrophages can potentially have a wide variety of inducer or suppressor functions in the lungs.⁵¹

The immunocytochemical studies for α GM-CSFr protein confirm that the increased number of mRNA transcripts being detected in the intrinsic asthma group are translated into receptor proteins, as previously described for other cell surface receptors.⁵² The differential expression of α GM-CSFr in patients with intrinsic asthma was significantly increased compared with that of control subjects for expression of both mRNA and protein. However, although the mean numbers of cells per millimeter of basement membrane expressing mRNA and protein for α GM-CSFr in intrinsic asthma were similar, there was no significant correlation between the two. The relatively poor correlation between mRNA and protein may be explained by the fact that there are several factors that have the capacity to independently regulate α GM-CSFr gene transcription and translation.

In conclusion, we have demonstrated evidence for elevated numbers of α GM-CSFr-positive macrophages in the bronchial tissue of patients with nonatopic asthma and have shown that there is an inverse correlation between α GM-CSFr expression and FEV₁. Our data

support the roles of GM-CSF and macrophages in nonatopic asthma.

We thank Elsa Schotman, Zivart Yasruel, and Marie Plourde for their invaluable technical assistance.

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