

Eosinophilic inflammation is associated with elevation of interleukin-5 in the airways of patients with spontaneous symptomatic asthma

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In vitro and in vivo studies have shown an important role for interleukin-5 (IL-5) in regulating eosinophil proliferation, survival, and effector function. Because eosinophilic inflammation is an important component of symptomatic episodes of asthma, we have investigated whether increased levels of IL-5 protein are present in bronchoalveolar lavage (BAL) fluid of patients with spontaneously symptomatic asthma (FEV₁, 61% predicted; FEF_{25%-75%}, 30% predicted) compared with patients with asymptomatic asthma (FEV₁, 88% predicted; FEF_{25%-75%}, 76% predicted). The percent of BAL eosinophils (10.5% vs 0.6%) ($p = 0.0001$) and eosinophil-derived neurotoxin (386.0 ng/ml vs 6.3 ng/ml) ($p = 0.0001$) was greater in BAL fluids derived from patients with symptomatic asthma compared with patients with asymptomatic asthma. Levels of IL-5 measured with an immunoradiometric assay were significantly higher in patients with symptomatic asthma ($n = 26$) compared with those with asymptomatic asthma ($n = 18$) (274 pg/ml vs <13 pg/ml) ($p = 0.02$). The increased IL-5 levels were noted in a subset of patients with symptomatic asthma with BAL absolute eosinophil counts greater than 10^6 (IL-5, 664 pg/ml; $n = 10$) as opposed to patients with symptomatic asthma with BAL eosinophil counts less than 10^6 (IL-5, <13 pg/ml; $n = 16$) ($p = 0.005$). This study suggests that IL-5 is not only induced in experimental models of allergen-induced asthma but can also be detected as asthma progresses from the asymptomatic to the clinically symptomatic state in subjects with significant BAL eosinophilia. (J ALLERGY CLIN IMMUNOL 1995;96:661-8.)

Key words: IL-5, eosinophil, asthma, eosinophil-derived neurotoxin

Cumulative evidence from *in vitro* analyses¹ and more recently *in vivo* animal^{2,3} and human studies^{4,5} indicates a link between interleukin-5 (IL-5) production and eosinophilia. For example, transgenic mice constitutively expressing the IL-5 gene

Abbreviations used

BAL:	Bronchoalveolar lavage
EDN:	Eosinophil derived neurotoxin
FEF _{25%-75%} :	Forced expiratory flow, mid-expiratory phase
IL-5:	Interleukin-5

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exhibit a significant tissue eosinophilia,² which can be inhibited by the *in vivo* administration of an antibody to the IL-5 receptor.⁶ In addition, neutralizing antibodies to IL-5 significantly inhibit the development of pulmonary eosinophilia in parasitized mice.⁷

Subsequent human studies have therefore sought to identify a similar association between IL-5 and eosinophilia in allergic diseases, such as asthma, which are characterized by tissue eosino-

TABLE I. Clinical characteristics and percent BAL eosinophils in patients with asthma

	Symptomatic asthma (<i>n</i> = 26)	Asymptomatic asthma (<i>n</i> = 18)
Age (yr)	42 ± 16 (22-67)	24 ± 6 (18-39)
Sex (M/F)	10/16	9/9
Smoker	0/26	0/18
Atopic	20/26	18/18
Percent FEV ₁ (range)	61 ± 10 (48-72)	88 ± 9 (75-101)
Percent FEF _{25%-75%} (range)	30 ± 15 (12-60)	76 ± 17 (46-101)
Percent BAL eosinophils (range)	10.5 ± 7.7 (1.4-3.6)	0.6 ± 1.0 (0-4.1)

Results are expressed as mean ± SD.

philia. Overall, these studies of asthma demonstrate that a small number of inflammatory cells expressing IL-5 mRNA are present in bronchoalveolar lavage (BAL) cells and mucosal biopsy specimens derived from patients with asymptomatic asthma and that the expression of IL-5 mRNA can be markedly induced by endobronchial instillation or inhalation of allergen.⁸⁻¹¹ In addition to the studies of IL-5 mRNA expression, studies of IL-5 protein have demonstrated measurable IL-5 protein levels in BAL fluids of patients with mild asthma¹² and induction of IL-5 protein in BAL fluids obtained 48 hours after endobronchial instillation of allergen in patients with allergic rhinitis.¹³ In contrast to the previously mentioned studies of patients with mild asthma or allergic rhinitis, there is currently no information on BAL levels of IL-5 protein in spontaneously symptomatic episodes of asthma of moderate severity. Because this group of patients with moderately symptomatic asthma is characteristic of many patients encountered in clinical practice, it is important to define whether IL-5 protein is expressed at higher levels in these patients with symptomatic asthma compared with patients with asymptomatic asthma.

To answer these questions we have tested the BAL fluids from patients with spontaneously symptomatic asthma of moderate severity (*n* = 26) and asymptomatic asthma (*n* = 18) for the presence of IL-5 with a sensitive immunoradiometric assay. We have focused on addressing the following questions related to IL-5 and asthma, which have not previously been addressed. (1) Is IL-5 protein elevated in BAL fluid obtained from patients with spontaneous symptomatic asthma of moderate severity? (2) Do BAL fluid IL-5 levels differ in patients with symptomatic asthma and those with asymptomatic asthma? (3) Do BAL fluid IL-5 levels correlate with BAL fluid eosino-

phil numbers and eosinophil granule protein levels in patients with symptomatic asthma?

METHODS

Patients

The patients' clinical data are summarized in Table I. All patients with asthma were nonsmokers and met the American Thoracic Society criteria for the diagnosis of asthma.¹⁴ Patients were classified as atopic on the basis of results of immediate hypersensitivity skin tests or IgE RAST with environmental allergens as previously described.¹⁵

Symptomatic asthma (*n* = 26)

Patients with FEV₁ less than 75% of predicted value who underwent bronchoscopy for diagnostic or therapeutic purposes in the pulmonary department at Alvarado Hospital, San Diego, California, made up the symptomatic asthma group. No patients with symptomatic asthma were specifically recruited for the study, and the use of the otherwise discarded BAL fluid was approved by the Human Subjects Committee of the University of California, San Diego. The use of bronchoscopy and lavage in this setting has previously been safely performed.^{16,17} Some of the patients with symptomatic asthma (*n* = 18 of 26) were the same as those previously described.¹⁵ Before bronchoscopy was performed, all patients with symptomatic asthma were treated as outpatients with various combinations of inhaled β-agonists, corticosteroids, cromolyn, or ipratropium bromide; oral theophylline; and oral or intravenous corticosteroids.¹⁵

Asymptomatic asthma (*n* = 18)

These patients had FEV₁ greater than 75% of predicted value without wheezing. All subjects with asymptomatic asthma had a positive methacholine challenge (provocative concentration causing a 20% fall in FEV₁ <8 mg/ml) and an increase in FEV₁ of 15% or more after inhalation of a β-agonist through a metered-dose inhaler.¹⁰ The BAL fluid from patients with asymptomatic asthma was obtained before endobronchial allergen challenge was done in a study approved by the Human

Subjects Committee of the University of California, San Diego. Some of these patients (5 of 18) have also been described in a previous study.¹⁰ Medications used by patients with asymptomatic asthma included inhaled β -agonists and oral theophylline on an as-needed basis. No subject with asymptomatic asthma had been treated in the emergency room or had been hospitalized for an asthma exacerbation in the 6 months before the study.

BAL and processing of BAL fluids

After topical anesthesia was induced, a 100 ml BAL was performed in the right lower lobe with a fiberoptic bronchoscope as previously described.¹⁵ All BAL fluids were centrifuged at 300 *g* for 10 minutes at 4° C to pellet the cells. The supernatant fluids were stored at -70° C for later IL-5 and eosinophil-derived neurotoxin (EDN) analysis. Cytocentrifuge preparations of the pelleted BAL cells were stained with Wright Giemsa stain. Differential cell counts were made by counting a minimum of 200 cells with a standard light microscope.

IL-5 immunoradiometric assay

A two-site immunoradiometric assay was performed to measure IL-5 in the BAL fluid. Immulon-4 wells were coated with a rat anti-human IL-5 monoclonal antibody (5A10) (Dr. John Abrams, DNAX Research Laboratories, Palo Alto, Calif.). Dilutions of standard amounts of recombinant human (rh)IL-5 (Dr. S. Narula, Schering Plough, Kenilworth, N.J.) or test BAL fluids were incubated overnight at room temperature. After the wells were washed, I¹²⁵-labeled IgG rabbit anti-human IL-5 polyclonal antibody was added to the wells. After incubation overnight at room temperature, the plates were washed and the wells analyzed for bound radioactivity in a Riasar multichannel gamma counter (Packard Instrument Co., Downers Grove, Ill.). The values of IL-5 in the test BAL fluids were determined with a calibration curve generated by varying dilutions of rhIL-5 standards. No cross-reactivity was seen in this assay with rhIL-3, rh granulocyte-macrophage colony-stimulating factor, and rhIL-4 (data not shown). The lower limit of IL-5 detection was 13 pg/ml. All values are expressed as picograms per milliliter in unconcentrated BAL fluid.

Measurement of EDN

EDN was measured with a competitive binding radioimmunoassay with a polyclonal antibody to EDN, as previously described.¹⁸ The sensitivity of the assay is 5 ng/ml.

Statistical analysis

Comparisons between BAL IL-5 (or EDN) levels of the symptomatic and asymptomatic asthma groups were performed by a Mann-Whitney U test. If an IL-5 or EDN level in a particular patient fell below the sensitivity of the respective assay, the lowest detectable value obtained for that assay (13 pg/ml for IL-5; 5 ng/ml for EDN) was arbitrarily used for statistical analysis. Statis-

tical comparisons between high and low IL-5 or eosinophil subgroups within the symptomatic asthma group were performed by a paired two-tailed *t* test. Correlations between IL-5 levels and cell type recruited were performed by simple (Pearson) correlation statistics. Results are expressed as mean $\pm$ SD unless otherwise indicated.

RESULTS

Pulmonary function and BAL fluid analysis

Table I shows the clinical characteristics of the asthma study groups. The FEV₁ (60.9% $\pm$ 9.7% vs 88.3% $\pm$ 9.0%) (*p* = 0.0001) and forced expiratory flow, mid-expiratory phase (FEF_{25%-75%}) (29.7% $\pm$ 14.6% vs 75.8% $\pm$ 16.9%) (*p* = 0.0001) were considerably lower in the symptomatic group compared with the asymptomatic group. The BAL fluid differential cell count in patients with symptomatic asthma (77.1% $\pm$ 10.5% alveolar macrophages, 8.0% $\pm$ 2.7% lymphocytes, 10.5% $\pm$ 7.7% eosinophils, 4.4% $\pm$ 3.0% neutrophils) revealed a statistically significant increase in BAL eosinophils (*p* = 0.0001) and neutrophils (*p* = 0.0001) compared with BAL cells recovered from patients with asymptomatic asthma (91.5% $\pm$ 3.0% alveolar macrophages, 7.9% $\pm$ 2.9% lymphocytes, 0.6% $\pm$ 1.0% eosinophils, 0.1% $\pm$ 0.1% neutrophils).

Levels of EDN were significantly elevated in BAL fluid derived from patients with symptomatic asthma (386.0 $\pm$ 167.8 ng/ml) compared with those with asymptomatic asthma (6.3 $\pm$ 0.8 ng/ml) (*p* = 0.0001) (Fig. 1). Levels of BAL eosinophils correlated significantly with EDN levels (*r* = 0.75, *p* = 0.0001). The FEV₁ in patients with asymptomatic asthma and those with symptomatic asthma showed an inverse correlation with BAL eosinophils (*r* = -0.69, *p* = 0.001), BAL EDN (*r* = -0.71, *p* = 0.001), and BAL IL-5 (*r* = -0.41, *p* = 0.01).

IL-5 levels are higher in patients with symptomatic asthma with BAL eosinophilia greater than 1×10^6

Fig. 2 shows the IL-5 levels and corresponding eosinophil counts in patients with symptomatic asthma and those with asymptomatic asthma. IL-5 was detected in seven of 10 patients with symptomatic asthma who had BAL absolute eosinophil counts (percent BAL eosinophils $\times$ total BAL cell count) greater than 100×10^4 (1×10^6) (Fig. 2, *B*). In contrast, IL-5 was not detected in any of 16 patients with symptomatic asthma (Fig. 2, *B*) and 18 patients with asymptomatic asthma (Fig. 2, *A*) who had BAL absolute eosinophil counts less than

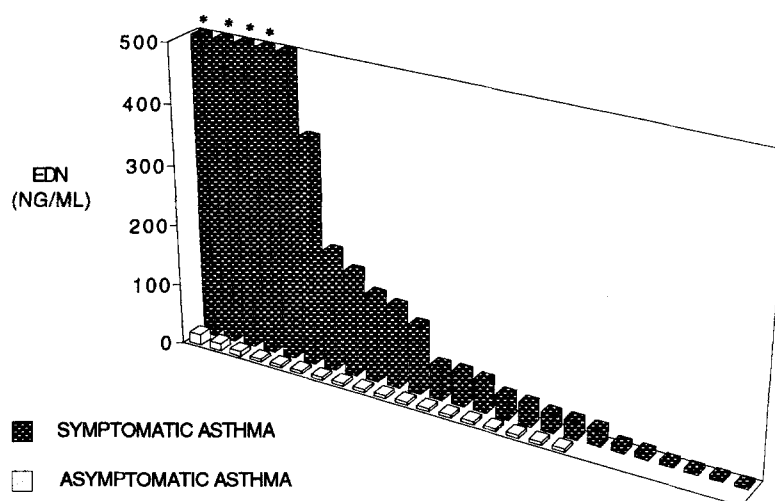


FIG. 1. BAL levels of EDN in patients with symptomatic and asymptomatic asthma. BAL fluids from patients with symptomatic and asymptomatic asthma were analyzed for the presence of EDN with a competitive binding radioimmunoassay. All values are expressed as nanograms per milliliter in unconcentrated BAL fluids. The four patients with symptomatic asthma depicted with * had EDN levels >500 ng/ml (3387 ng/ml, 2700 ng/ml, 941 ng/ml, and 737 ng/ml).

100×10^4 . The IL-5 levels in patients with symptomatic asthma, which were 274 ± 232 pg/ml ($n = 26$), were higher than those in patients with asymptomatic asthma, which were $<13 \pm 0$ pg/ml ($n = 18$), ($p = 0.02$). Both allergic (6 of 20) and nonallergic (1 of 6) patients with symptomatic asthma ($n = 26$) had detectable IL-5 levels. The IL-5 levels noted in the subset of patients with symptomatic asthma with BAL absolute eosinophil counts greater than 10^6 (IL-5, 664 ± 575 pg/ml; $n = 10$) were significantly greater than IL-5 levels in patients with symptomatic asthma with BAL eosinophil counts less than 10^6 (IL-5, <13 pg/ml; $n = 16$) ($p = 0.005$).

Table II shows differences in the patients with symptomatic asthma who had high levels of BAL eosinophilia ($>10^6$ BAL eosinophils) ($n = 10$) compared with the patients with symptomatic asthma who had low levels of BAL eosinophilia ($<10^6$ BAL eosinophils) ($n = 16$). Significantly higher levels of EDN ($p = 0.03$), eosinophils ($p = 0.0001$), neutrophils ($p = 0.002$), and total cells ($p = 0.03$) but not lymphocytes ($p = 0.24$) or alveolar macrophages ($p = 0.08$) were present in the high compared with the low BAL eosinophil subgroup. The high BAL eosinophil subgroup had a significantly lower $FEF_{25\%-75\%}$ ($p = 0.05$), but not FEV_1 ($p = 0.44$), compared with the low BAL eosinophil subgroup.

IL-5 levels correlated to the number of eosinophils ($r = 0.84$, $p = 0.0001$), neutrophils ($r = 0.84$, $p = 0.0001$), lymphocytes ($r = 0.69$, $p = 0.0001$),

and the total number of BAL cells ($r = 0.53$, $p = 0.007$), but not to the number of alveolar macrophages ($r = 0.23$, $p = 0.27$). IL-5 also correlated to levels of EDN ($r = 0.48$, $p = 0.001$).

DISCUSSION

In this study IL-5 levels were significantly higher in patients with spontaneously symptomatic asthma (FEV_1 , 61% predicted; $FEF_{25\%-75\%}$, 30% predicted) when compared with undetectable IL-5 levels in those with asymptomatic asthma (FEV_1 , 88% predicted; $FEF_{25\%-75\%}$, 76% predicted). In addition, the percent of BAL eosinophils (10.5% vs 0.6%) and levels of the eosinophil granule protein EDN (386.0 ng/ml vs 6.3 ng/ml) were greater in BAL fluids derived from patients with symptomatic asthma compared with BAL fluids from those with asymptomatic asthma.

This study differs from previous studies of IL-5 and asthma^{4, 5, 8-10, 12, 13} in that it demonstrates for the first time: (1) differences in levels of BAL IL-5 protein in patients with symptomatic asthma and those with asymptomatic asthma and (2) an association between BAL IL-5 protein levels and BAL eosinophils and eosinophil granular proteins in spontaneous asthma of moderate severity. In addition, we have used a specific IL-5 immunoassay, as opposed to an IL-5 bioassay¹² (which may nonspecifically detect cytokines other than IL-5 present in BAL fluid, unless the bioassay results are verified with a neutralizing antibody to IL-5), to conclusively demonstrate the presence of IL-5 in

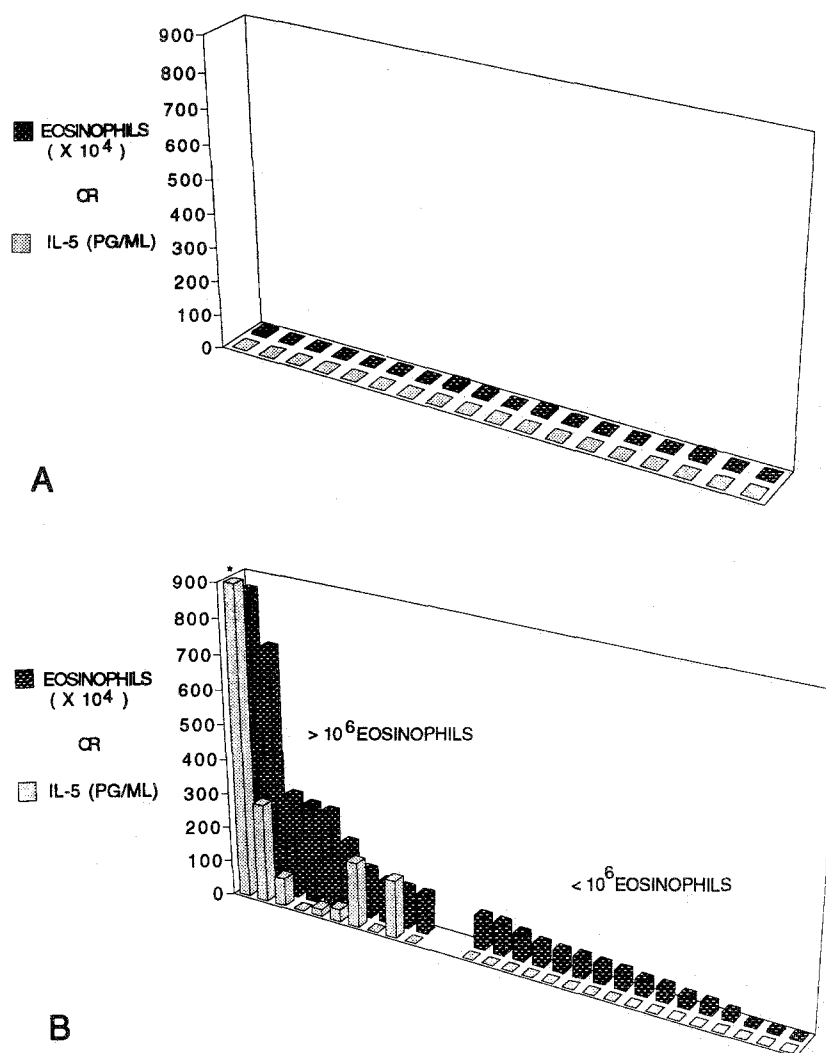


FIG. 2. BAL levels of IL-5 and absolute number of eosinophils in patients with asymptomatic (A) and symptomatic asthma (B). BAL fluids from patients with symptomatic asthma ($n = 26$) and asymptomatic asthma ($n = 18$) were analyzed for presence of IL-5 by an immunoradiometric assay and expressed as picograms per milliliter in unconcentrated BAL fluids. The subject with symptomatic asthma indicated with an *asterisk* had an IL-5 level of 5835 pg/ml. The corresponding number of BAL eosinophils for each subject in the study is also indicated.

BAL fluid and to demonstrate differences in amounts of BAL IL-5 in patients with symptomatic asthma and those with asymptomatic asthma.

The absence of detectable IL-5 in all subjects with symptomatic asthma may relate to previous therapy, IL-5 assay sensitivity, or subsets of patients with asthma who do not express IL-5. Although the mean age of the patients with symptomatic asthma (42 ± 16 years) was greater than the age of patients with asymptomatic asthma (24 ± 6 years), age alone is unlikely to account for elevations in cytokine levels. This conclusion is suggested from the subset of patients with symptomatic asthma who were younger than 35 years of

age (mean age, 26 ± 8 years; $n = 10$); three of them had elevated IL-5 levels (data not presented).

The study design we have used (comparing two different groups of subjects with asthma based on symptoms) is also unlikely to account for the detection of IL-5 in only the symptomatic group. We have recently determined BAL IL-5 levels in a second group of patients with asymptomatic asthma, ($n = 6$) studied at the Mayo Clinic, and we have not detected measurable BAL IL-5 in these patients. Thus the patients with asymptomatic asthma recruited at the University of California, San Diego, are unlikely to be a nonrepresentative study population.

TABLE II. Comparison between high and low BAL eosinophil subgroups of patients with symptomatic asthma and patients with asymptomatic asthma

	Symptomatic asthma			p Value		
	BAL Eosinophil >10 ⁶ cells (n = 10)	BAL Eosinophil <10 ⁶ cells (n = 16)	Asymptomatic asthma (n = 18)	1* vs 2†	2 vs 3‡	1 vs 3
Eosinophils (× 10 ⁴)	312.4 ± 83.4	46.3 ± 7.1	2.5 ± 1.0	0.0001	0.0001	0.0001
Neutrophils (× 10 ⁴)	100.8 ± 26.8	30.0 ± 7.6	0.5 ± 0.3	0.002	0.0001	0.0001
Lymphocytes (× 10 ⁴)	122.1 ± 31.2	68.9 ± 10.5	43.9 ± 8.6	NS	0.05	0.01
Macrophages (× 10 ⁴)	1114.8 ± 179.0	692.2 ± 118.9	558.6 ± 94.2	NS	NS	0.02
Total cells (× 10 ⁵)	165.0 ± 26.7	83.7 ± 13.8	61.1 ± 10.2	0.03	NS	0.001
FEV ₁ (%)	57.7 ± 2.3	61.2 ± 2.9	88.3 ± 2.1	NS	0.0001	0.0001
FEF _{25%-75%} (%)	22.6 ± 3.3	35.2 ± 4.2	75.8 ± 4.0	0.05	0.0001	0.0001
EDN (ng/ml)	656.7 ± 358.2	233.8 ± 166.1	6.3 ± 0.8	0.03	0.0001	0.0001
IL-5 (pg/ml)	664 ± 575	<13	<13	0.005	NS	0.003

Results are expressed as mean ± SEM.

*1 = column 1; BAL eosinophils >10⁶ cells in symptomatic asthma.

†2 = column 2; BAL eosinophils <10⁶ cells in symptomatic asthma.

‡3 = column 3; asymptomatic asthma.

NS, Not significant.

In this study we were unable to address whether the symptomatic state or corticosteroid treatment associated with the symptomatic state (each patient with symptomatic asthma received at least one dose of corticosteroid before BAL) was responsible for the generation of cytokines that were detected in BAL fluid. The previous detection of cytokines other than IL-5 in the BAL fluid of patients with symptomatic asthma not treated with corticosteroids¹⁹ and the ability of corticosteroids to inhibit the generation of IL-5 in vitro²⁰ would suggest that the symptomatic state, and not corticosteroid therapy associated with the symptomatic state, was responsible for the presence of IL-5 detected in BAL fluid. Corticosteroid therapy may also account for the absence of detectable IL-5, eosinophils, or EDN in BAL fluid of many patients with symptomatic asthma in this study.

IL-5 was not detectable in the subset of patients with symptomatic asthma ($n = 0$ of 16) who had low levels of eosinophil inflammation (<10⁶ total BAL eosinophils), but it was detected in the subset of patients with symptomatic asthma ($n = 7$ of 10) who had high levels of eosinophilic inflammation (>10⁶ total BAL eosinophils). The levels of IL-5 correlated both with the number of BAL eosinophils and with EDN levels. Sedgwick et al.¹³ have previously demonstrated a correlation between BAL IL-5 protein, eosinophils, and eosinophil granular proteins in subjects with allergic rhinitis who underwent segmental allergen challenge. The results of our study extend their observations,

developed in a model of allergen-induced airway inflammation,¹³ to patients with clinically symptomatic spontaneous episodes of asthma.

Walker et al.¹² have previously measured IL-5 protein in BAL fluids of patients with allergic (mean FEV₁, 88% predicted) and nonallergic asthma (mean FEV₁, 86% predicted).¹² Although the mean FEV₁ in allergic patients with asthma (88%) and nonallergic patients with asthma (86%) in that study indicated that the severity of the disease was mild, the range of FEV₁ in allergic patients with asthma (40% to 127%) and nonallergic patients with asthma (44% to 116%) studied indicated that they included in their analysis some subjects we have classified as patients with symptomatic asthma (FEV₁ <75%), and this factor could account for their detection of IL-5 in patients with mild asthma. In that study¹² IL-5 was measured with an IL-5 bioassay using the IL-5-sensitive LyH7B13 cell line, and results were expressed in IL-5 units per milliliter. Because we have expressed our IL-5 levels in picograms per milliliter, we are not able to directly compare the mean levels of IL-5 detected in our patients with spontaneously symptomatic asthma (274 pg/ml) with the levels of IL-5 previously reported in allergic patients with asthma (2.7 U/ml), nonallergic patients with asthma (2.1 U/ml), and normal healthy control subjects (0.6 U/ml). The previous detection of IL-5 in the normal control subjects and patients with mild asthma may

relate either to the IL-5 bioassay detecting cytokines other than IL-5 (neutralizing antibodies to IL-5 were not used in the IL-5 bioassay to confirm that all the bioactivity detected in BAL fluid was indeed IL-5), their inclusion of both patients with mild asthma and those with moderate asthma in one group (we have separated groups of patients with asthma based on FEV₁, FEF_{25% to 75%}, and symptoms), the sensitivity of the IL-5 bioassay used, or to the 20-fold concentration of BAL fluid before bioassay (BAL fluid was not concentrated in our study).

The levels of IL-5 correlated to the numbers of eosinophils, lymphocytes, neutrophils, and the total number of cells in this study. A correlation between IL-5 levels and eosinophil number has previously been demonstrated after segmental allergen bronchoprovocation in patients with allergic rhinitis.¹³ Correlation between IL-5 and the number of eosinophils or lymphocytes could either be due to production of IL-5 by eosinophils,¹⁰ T cells,⁹ or another cell type such as mast cells.²¹ In addition to correlations with eosinophils and lymphocytes, IL-5 levels correlated with the number of neutrophils. In contrast to lymphocytes and eosinophils, neutrophils are not a known source of IL-5 mRNA or protein. Because several cytokines, in addition to IL-5, are present in BAL fluid of patients with asthma,^{12, 15} another such cytokine (i.e., IL-8 or other neutrophil active cytokine), produced in association with IL-5, could account for the correlation. Therefore although correlations of a cytokine with a particular cell type in BAL fluid may be instructive in terms of suggesting cellular sources of cytokines or target cell action of the cytokine, the potential for correlations to have no cause and effect relationship should always be considered.

In summary, elevated levels of IL-5 were detected in patients with spontaneous symptomatic asthma of moderate severity but not in patients with asymptomatic asthma. In addition, levels of IL-5 correlated with the degree of eosinophilic inflammation and the levels of the eosinophil granule protein EDN. This study suggests that IL-5 is not only induced in experimental models of allergen-induced asthma¹³ but can also be detected in spontaneous clinical asthma as it progresses from the asymptomatic to the symptomatic state in subjects with significant BAL eosinophilia.

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