Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma

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Background: *We recently demonstrated that T lymphocytes in bronchoalveolar lavage (BAL) fluid from atopic asthmatic patients were activated and expressed increased cytokine messenger ribonucleic acid (mRNA) for "T_{H2}-type" cytokines, particularly IL-4 and IL-5, when compared with those in normal control subjects. This pattern of cytokines may determine the nature of the cellular infiltrate in the bronchial mucosa in asthma and hence the bronchial hyperresponsiveness (BHR) and symptoms that characterize this condition.*
Methods: *To examine the association between these cytokines and clinical measures of asthma severity we have extended our studies of BAL cells from subjects with atopic asthma. Numbers of BAL cells with positive in situ hybridization signals for IL-2, IL-3, IL-4, IL-5, granulocyte macrophage colony-stimulating factor (GM-CSF), and interferon-γ were counted on cytocentrifuge preparations. Results were compared between patients with symptomatic* ($n = 19$) *and asymptomatic asthma* ($n = 10$), *and associations were sought with airway methacholine responsiveness, resting airway caliber, and asthma symptom scores.*
Results: *There were increased proportions of cells positive for IL-3* ($p < 0.05$), *IL-4* ($p < 0.005$), *IL-5* ($p < 0.005$), *and GM-CSF* ($p < 0.005$) *mRNA in BAL fluid from patients with symptomatic asthma when compared with that from subjects free of symptoms, but no difference between the groups in numbers of cells expressing IL-2 and interferon-γ mRNA. There were significant associations among numbers of cells expressing mRNA for IL-4, IL-5, and GM-CSF, and airflow restriction, BHR, and Aas asthma score.*
Conclusions: *These findings support the hypothesis that cytokines contribute to airway events that determine asthma symptoms and BHR. (J ALLERGY CLIN IMMUNOL 1993;92:397-403.)*

Key words: *Asthma symptoms, bronchial hyperresponsiveness, bronchoalveolar lavage, T lymphocytes, interleukins*

Recent evidence suggests that activation of T-helper lymphocytes may have a role in the pathogenesis of atopic asthma.[1-3] In situ hybridization showed increased numbers of cells positive for messenger ribonucleic acid (mRNA) encoding IL-2, IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in bronchoalveolar lavage (BAL) fluid from atopic patients with asthma when compared with those from healthy volunteers, but no difference between the

Abbreviations used

BAL:	Bronchoalveolar lavage
BHR:	Bronchial hyperresponsiveness
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
IFN-γ:	Interferon-γ
mRNA:	Messenger ribonucleic acid
PEFR:	Peak expiratory flow rate

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two groups in cells expressing mRNA for interferon-γ (IFN-γ).[4] IL-4 and IL-5 mRNA was predominantly localized to T cells within BAL fluid from asthmatic subjects.[4] In vitro and animal studies suggest that these cytokines may be involved in maturation and activation of eosinophils and basophils,[5-9] and that IL-4, unopposed by IFN-γ, promotes B cell IgE synthesis.[10] Allergen-induced IgE-dependent mast-cell degranulation may be responsible for acute bronchoconstrictor responses in atopic asthma,[11] and eosinophil infiltration and activation may contribute to more chronic bronchial hyperreactivity.[12, 13] We therefore hypothesized that expression of mRNA for these cytokines in BAL fluid from atopic asthmatic subjects might relate to the level of bronchial hyperresponsiveness (BHR) and asthma symptoms. The aim of the current study was to extend our previous patient group[1, 4] to allow examination of associations among the proportion of BAL cells that express cytokine mRNA and asthma severity as measured by airway caliber, BHR, and asthma symptoms in a group of atopic asthmatic subjects.

METHODS

Asthmatic subjects were recruited from the Allergy Clinic of the Royal Brompton National Heart and Lung Hospital, as previously described.[1, 4] The study was approved by the local ethics committee, and all subjects gave written informed consent. Clinical characteristics of these subjects have been described previously, together with analysis of their BAL fluid T-lymphocyte activation status.[1] Asthma was defined by a clinical history of wheeze, cough, or breathlessness accompanied by documented reversible airflow limitation (20% or more variability of peak expiratory flow rate or FEV_1) and/or increased airway methacholine responsiveness (methacholine PC_{20} of less than 8 mg/ml). None of the patients was a smoker and none had received oral or inhaled corticosteroids in the 3 months before the study. All had a FEV_1 greater than 50% of predicted, and any subject with a history suggestive of respiratory infection in the preceding 4 weeks was excluded.

History and examination was performed and patients' atopic status was assessed by skin prick tests (Soluprick, ALK Horsholm, Denmark) and measurement of total and specific IgE (RAST, Pharmacia, Uppsala, Sweden) to common aeroallergens as previously described.[1] Subjects were divided into those with current symptoms who required use of inhaled bronchodilators at least twice per week in the 2 weeks preceding the study and those subjects free of symptoms with seasonal or strictly allergen-related symptoms only who had been free of symptoms for at least 4 weeks before the study.[1] Symptom scores were allocated by the method described by Aas.[14] Spirometry was recorded with a dry wedge bellows spirometer (Vitalograph, Bucks, United Kingdom), and methacholine PC_{20} was measured at least 12 hours after the last dose of any inhaled β_2-agonist with a tidal breathing method.[15]

Forty-eight hours after the initial assessment subjects underwent fiberoptic bronchoscopy, which was performed between 8 and 9 AM after premedication with salbutamol by nebulizer in all cases. Bronchoscopy was done as previously described,[1, 4] and BAL was performed by instillation of four 60-ml aliquots of warmed, pH-balanced sterile saline into the right middle lobe or lingula, with immediate gentle aspiration into a siliconized glass bottle as previously described.[1, 4]

Cells from the BAL fluid were filtered through a double layer of sterile cotton gauze and centrifuged at 400 *g* for 7 minutes at 4° C, then resuspended in 1.5 ml RPMI 1640 medium (Flow Laboratories, Irvine, Scotland). Cells were adjusted to a concentration of 5×10^5 per milliliter, and cytocentrifuge preparations were made as previously described.[1, 4] For in situ hybridization slides were precoated with poly-l-lysine (molecular weight >150,000, Sigma Chemicals, Poole, United Kingdom). After cytocentrifugation at 800 rpm for 5 minutes slides were air-dried for 5 minutes, then either stained with May-Grünwald Giemsa stain (Accustain, Sigma, St. Louis, Mo.) for differential cell count or fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4, BDH Chemicals, Poole, United Kingdom) for 30 minutes followed by two changes of 15% sucrose in phosphate-buffered saline (Sigma) before in situ hybridization studies.

Riboprobes (labeled RNA) for cytokine mRNA were prepared from complementary DNA for IL-2, IL-3, IL-4, IL-5, GM-CSF, and IFN-γ as previously described.[4] The protocol for prehybridization, hybridization, and washing was as previously described,[4] except that incubation in 0.1 mol/L triethanolamine and 0.1 mol/L N-ethylmaleimide (Sigma) was included in the prehybridization protocol, and 0.1 mol/L dithiothreitol (Sigma) was added to the hybridization mixture to prevent nonspecific binding of ^{35}S-labeled riboprobes. Negative control slides were hybridized with sense probes (identical sequence to mRNA) or pretreated with ribonuclease A (Promega, Southampton, United Kingdom). A T cell clone known to produce IL-3, IL-4, IL-5, and GM-CSF was used as a positive control for mRNA for these cytokines[4] and T cell blasts for IL-2 and IFN-γ. Autoradiography was done as described.[4] Quantification was done by counting of cells overlain by dense deposits of silver grains. This was performed by an observer blinded to clinical status, and triplicate counts were done.

Comparison of numbers of BAL cells per 1000 total cells expressing signals for cytokine mRNA in BAL fluid from asymptomatic subjects with those from subjects with current asthma symptoms was performed by the Mann-Whitney U test. Clinical measurements for

the two groups were also compared by Mann-Whitney U test. Correlations between clinical measures of asthma severity and the number of cells expressing positive hybridization signals for cytokine mRNA were by Spearman's rank correlation. All statistical manipulations were performed with Minitab software (Minitab Inc., State College, Pa.).

RESULTS

Nineteen subjects with asthma had current symptoms and 10 were free of symptoms at the time of the study. Clinical details are shown in Table I. Comparison of the two groups showed that those with current symptoms had significantly reduced FEV_1 ($p < 0.005$), lower PC_{20} ($p < 0.005$), and a higher symptom score ($p < 0.01$) than subjects with asthma who were free of symptoms.

Fiberoptic bronchoscopy was well tolerated by all subjects, and no complications from the procedure were observed. No differences were observed between the two groups of patients with asthma for the total volume of BAL fluid recovered nor in the total cell yield for the subjects with and without symptoms, respectively (Table II). The median percentage of eosinophils in BAL fluid from the patients with symptomatic asthma was higher than that for those with asymptomatic asthma although this did not achieve statistical significance (Table II).

In situ hybridization studies revealed increased numbers of BAL cells per 1000 with positive signals for IL-3 ($p < 0.05$), IL-4 ($p < 0.005$), IL-5 ($p < 0.005$), and GM-CSF ($p < 0.005$) mRNA in BAL fluid from subjects with symptoms when compared with BAL fluid from those without symptoms (Fig. 1). There was no difference between the two groups in numbers of BAL cells per 1000 positive for IL-2 ($p = 0.1$) or IFN-γ mRNA ($p = 0.2$).

When all 30 subjects with asthma were considered there was a significant association between numbers of BAL cells per 1000 expressing mRNA for IL-5, FEV_1, methacholine PC_{20} (Fig. 2), and Aas symptom score ($r_s = 0.41$, $p < 0.05$). There were also significant associations between numbers of cells per 1000 positive for IL-4 mRNA and FEV_1 ($r = -0.45$, $p < 0.01$) and Aas score ($r = 0.43$, $p < 0.01$). There was a significant negative correlation between airways responsiveness to methacholine and numbers of BAL cells per 1000 expressing mRNA for GM-CSF ($r = -0.44$, $p < 0.02$) and a positive correlation with the numbers expressing IFN-γ mRNA ($r = 0.48$, $p < 0.01$). There was also a correlation between the numbers of BAL cells per 1000 expressing mRNA for IL-4 and those expressing IL-5 mRNA ($r = 0.84$, $p < 0.001$, Fig. 2).

TABLE I. Clinical details of subjects with asthma studied

	Patients with symptomatic asthma	Patients with asymptomatic asthma
Age (yr)	24 (22-27)	26 (19-35)
Sex (F:M)	14:5	5:5
FEV_1 (%)	82 (62-108)	103 (84-129)*
PC_{20} (mg/ml)	0.6 (0.2-3.2)	10.4 (2.2-19.9)*
Aas score	3 (2-4)	1 (1-2)†

Value are medians with range.
*$p < 0.005$.
†$p < 0.01$.

The associations among BAL fluid eosinophils and numbers of cells expressing mRNA for IL-5 ($r = 0.13$), IL-3 ($r = 0.20$), and GM-CSF ($r = 0.18$) were not statistically significant.

DISCUSSION

In this study we have demonstrated increased numbers of BAL cells expressing mRNA for IL-3, IL-4, IL-5, and GM-CSF in subjects with symptomatic asthma when compared with those for subjects with asthma who did not have current symptoms but no difference between the two groups in cells expressing IL-2 and IFN-γ mRNA. Furthermore, associations were observed between the numbers of cells expressing mRNA for IL-4, IL-5, and GM-CSF and the clinical manifestations of asthma. These findings support the hypothesis that a "T_{H2}-type" pattern of cytokines may contribute to bronchial mucosal inflammation in asthma and are compatible with the suggestion that production of these cytokines may, at least in part, determine severity of asthma symptoms and BHR.

The proportion of cells that give positive in situ hybridization signals does not necessarily equate with the amount of mRNA for a particular cytokine present within the sample, still less with the level of active cytokine product. However, we have previously demonstrated marked differences in the numbers of BAL cells that give positive in situ hybridization signals for IL-4, IL-5, and GM-CSF mRNA from subjects with asthma and control subjects.[4] The recent detection of IL-4 and IL-5 in concentrated BAL fluid from atopic patients with asthma but not healthy control subjects suggests

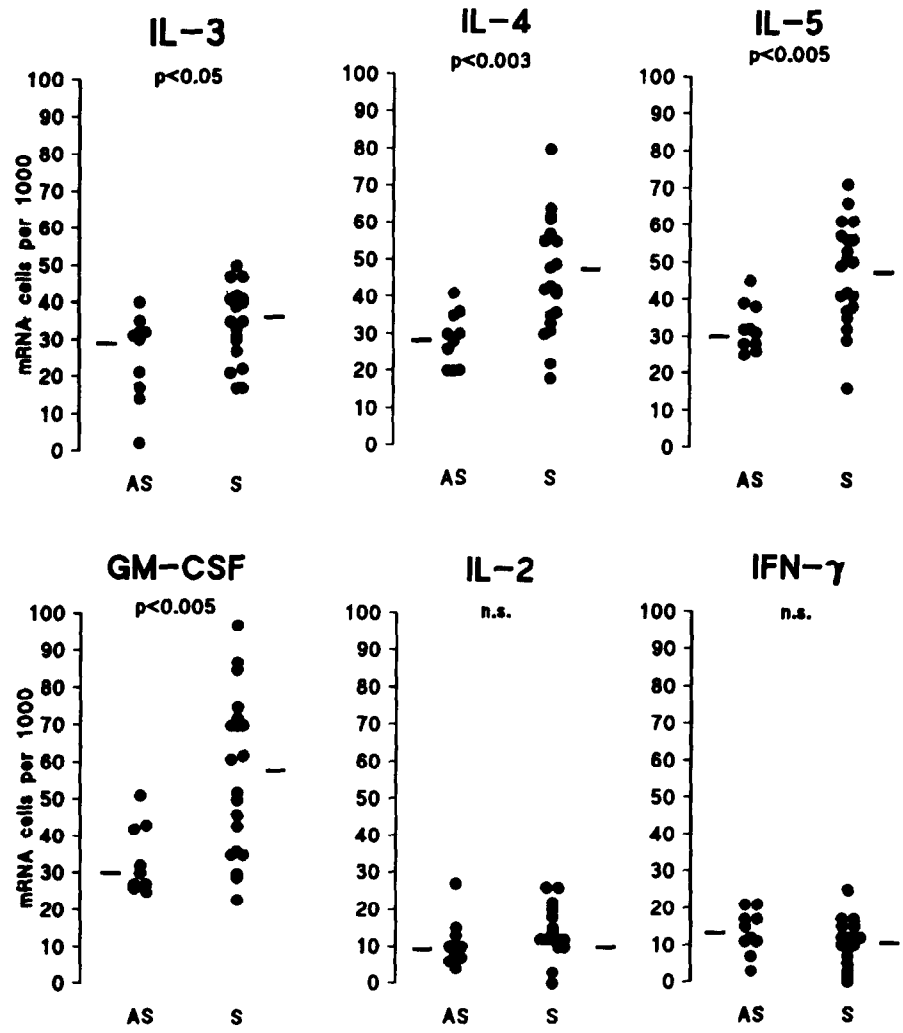

FIG. 1. Numbers of BAL cells per 1000 giving positive in situ hybridization signals for cytokine mRNA from subjects without symptoms (*AS*, $n = 10$) and from subjects with symptomatic asthma (*S*, $n = 19$). Median bars are indicated. *n.s.*, Not significant.

TABLE II. Volume of BAL fluid returned, total cell yield, and percentage cell counts from subjects with asymptomatic and symptomatic asthma

	Symptomatic asthma	Asymptomatic asthma	Estimated median difference (95% CI)	*p* Value
Volume (ml)	115 (60-150)	130 (95-150)	−15.0 (−35-10)	0.32
Total cells ($\times 10^6$)	10.1 (2.6-30.8)	12.5 (5.5-24.3)	−2.2 (−10.2-2.7)	0.42
Alveolar macrophages (%)	84.6 (13.8-90.2)	82.0 (64.7-88.4)	0.85 (−5.8-7.1)	0.71
Lymphocytes (%)	10.7 (7.2-23.5)	13.3 (10.1-20.8)	−3.0 (−6.6−−0.2)	0.03
Eosinophils (%)	2.25 (0.1-75.4)	0.85 (0.2-23.7)	1.2 (−0.5-5.1)	0.15
Neutrophils (%)	0.5 (0.0-5.2)	0.4 (0.0-1.2)	0.0 (−0.4-0.5)	1.0
Epithelial cells (%)	0.2 (0.0-10.0)	0.0 (0.0-0.4)	0.2 (0.0-0.5)	0.009

Values given are medians with ranges and estimated median differences between groups with 95% confidence intervals (*CI*).

that these differences are reflected in the translated cytokine product.[16] Similarly, we have recently reported increased numbers of BAL cells expressing mRNA for IL-4, IL-5, and GM-CSF 24 hours after allergen inhalation challenge of atopic subjects when compared with those of control challenge subjects,[17] and these findings are supported by the detection of IL-5 in BAL fluid after

segmental airway challenge of atopic nonasthmatic subjects[18] and the demonstration of immunoreactivity for GM-CSF together with positive mRNA hybridization signals in BAL cells obtained by fiberoptic bronchoscopy after segmental allergen challenge.[19]

The percentage of lymphocytes in BAL fluid from subjects with symptomatic asthma was slightly reduced when compared with that from those without symptoms, which suggests that the increase in numbers of cells positive for cytokine mRNA in the symptomatic group did not simply reflect a change in lymphocyte numbers. Although there was a trend for an increase in BAL fluid eosinophils in patients with symptomatic versus asymptomatic asthma, this difference was not statistically significant. We also did not find significant associations between percentage BAL fluid eosinophils and the proportions of cells expressing mRNA for eosinophil-acting cytokines. It is likely that the percentage of eosinophils detected in BAL by histochemical stains does not accurately reflect their state of activation or the levels of eosinophil granule proteins or lipid mediators that they produce.[20] The clinical expression of asthma symptoms is likely to reflect a complex interaction of mediators from many cell types, and although cytokines may contribute to the pattern of inflammatory response observed in atopic asthma this may not be reflected at the level of any one individual cell type.

In this study we have not identified the cell type responsible for positive in situ hybridization signals with BAL procedures in these patients. Although we previously reported a predominant T-cell source for IL-4 and IL-5 mRNA, a number of other cells, including mast cells,[21] eosinophils,[22, 23] and macrophages[24] may also produce cytokines. It will be important to identify the relative contributions of these cell types if cell-directed therapy is considered. The close association observed between numbers of cells positive for IL-4 mRNA and those expressing IL-5 mRNA is compatible with coregulation of mRNA encoding these cytokines in atopic asthma, as has been suggested in helminthic disease.[25] Because we were not able to determine whether the same cells express mRNA for different cytokines, we could not correlate total numbers of cytokine mRNA-positive cells with asthma severity. This would require simultaneous in situ hybridization with different probes, which would also be important in confirming whether the cytokine profile of individual cells does indeed conform to a T_{H2} pattern. The observed correlations between measures of asthma severity and numbers of BAL cells expressing cytokine mRNA would support the view that a T_{H2} pattern of cytokine expression is contributing to airflow obstruction and bronchial responsiveness in atopic asthma, rather than simply reflecting the atopic state. Nonetheless, it will be of interest to extend these studies to atopic subjects who do not have asthma. The positive

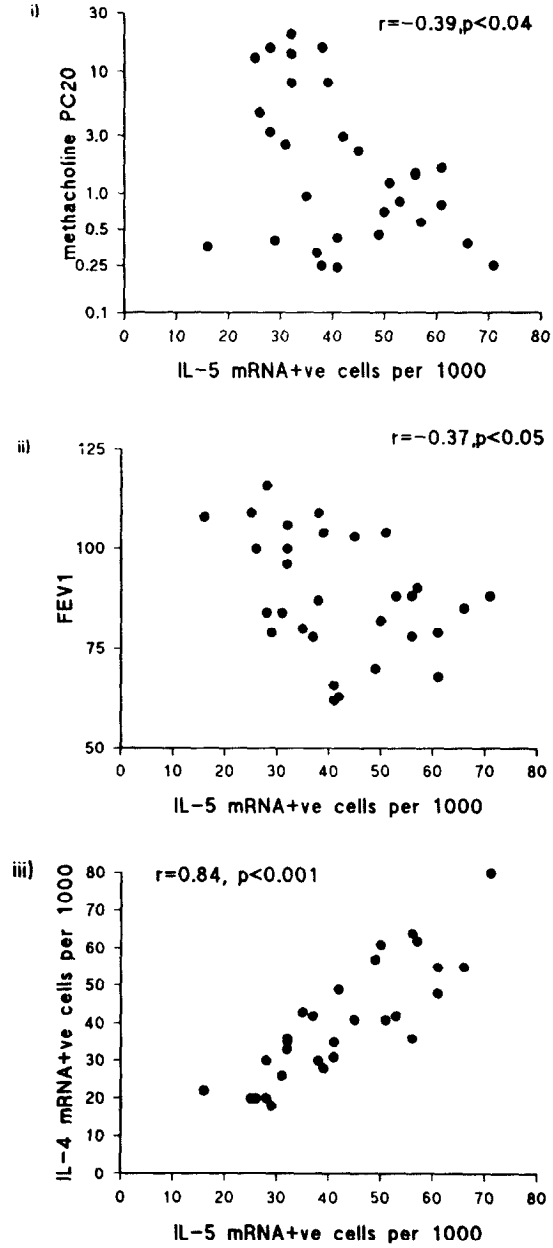

FIG. 2. Associations between numbers of BAL cells expressing cytokine mRNA and clinical measures of asthma. **i,** Relationship between cells positive for IL-5 mRNA and airway methacholine responsiveness (log PC_{20}). **ii,** Relationship between cells positive for IL-5 mRNA and FEV_1. **iii,** Relationship between numbers of cells expressing mRNA for IL-5 and numbers expressing mRNA for IL-4. *+ve,* Positive.

correlation observed between numbers of BAL cells positive for IFN-γ and the methacholine PC_{20} is of interest inasmuch as this cytokine is inhibitory both to IgE synthesis[10] and expansion of the T_{H2} subtype of T helper cells.[26] If a T_{H2}-equivalent T-cell population is present in atopic allergic disease,[27, 4] it may be possible to use the differences in intracellular signaling and activation requirements observed at the level of T_{H2} and T_{H2} clones to selectively inhibit activation of these cells.[28, 29]

Although the observed associations do not prove a causal link between cytokine production and bronchial mucosal inflammation these results do support the hypothesis that cytokines contribute airway events that determine asthma symptoms and BHR. This raises the possibility of novel therapeutic approaches directed at inhibition of cytokine synthesis or activity. Anti-IL-5 monoclonal antibodies inhibited the airway eosinophilia in an animal model of allergen-induced late responses.[30] Blocking antibody to IL-4 receptors protected against rejection of cardiac allografts in experimental animals.[31] Although initial reports of the use of systemic IFN-γ for allergic disease were disappointing,[32] the application of topical therapy would be of interest. Such approaches may allow specific asthma therapy without the adverse effects associated with less-specific immunosuppressive therapy such as the use of corticosteroids or cyclosporine A.[33]

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