

Granulocyte macrophage colony-stimulating factor is the main cytokine enhancing survival of eosinophils in asthmatic airways

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ABSTRACT: Interleukin (IL)-3, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF) prolong the survival of eosinophils, which are conspicuous in asthmatic airways, but it is still controversial which one plays a major role in enhancing the survival of eosinophils in asthmatic airways.

The role of these cytokines in airway eosinophilia was investigated using bronchoalveolar lavage (BAL) fluids from 11 symptomatic and nine asymptomatic patients with asthma and eight normal subjects. Eosinophil survival-enhancing activity (ESEA) was measured by a numerical change in viable eosinophils isolated from the peripheral blood of atopic patients and cultured with BAL fluids. ESEA was characterized by neutralization with antibodies to IL-3, IL-5 and/or GM-CSF. The differential count of BAL cells was achieved using Diff-Quik stain. T-cell subsets and activated T-cells were analysed by flow cytometry with dual stain using monoclonal antibodies to CD3, CD4, CD8 and CD25.

ESEA was detected in eight of 11 BAL fluids of symptomatic asthma, but not in those of normal controls or asymptomatic asthmatics. In six symptomatic asthmatics, the mean percentage of inhibition in ESEA by anti-GM-CSF was higher than that of anti-IL-5 as well as anti-IL-3 ($p < 0.05$). A mixture of antibodies to IL-3, IL-5 and GM-CSF totally inhibited the ESEA in four cases. The ESEA correlated with the percentage of eosinophils ($p < 0.05$) and that of CD25(+)CD4 lymphocytes ($p < 0.05$) of BAL cells.

In conclusion, granulocyte macrophage colony-stimulating factor, rather than interleukin-3 or -5, is associated with eosinophil survival-enhancing activity inside the airways of symptomatic asthmatics. The activation of CD4 lymphocytes is related to the elevation of such activity.

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Bronchial asthma is characterized as an eosinophilic inflammatory airway disease. Eosinophils increase in bronchoalveolar lavage (BAL) and the bronchial mucosa of symptomatic patients with asthma [1, 2]. Eosinophils are also prominent among BAL cells during the late asthmatic reaction after bronchial challenge with specific allergens [3, 4]. Eosinophils release mediators to induce airway inflammation and hyperreactivity [5]. Processes of adherence, chemotaxis and prolongation of survival determine the infiltration of eosinophils into the tissues. Once eosinophils are recruited into the bronchial mucosa from vessels, the prolongation of survival may be a major determinant of the infiltration of eosinophils. Among the eosinophil-active cytokines, interleukin (IL)-3, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF) are able to prolong the survival of eosinophils [6–9]. Messenger ribonucleic acid (mRNA) and proteins of IL-3, IL-5 and GM-CSF are expressed in the bronchial tree after specific allergen challenges [10, 11] and during symptomatic periods of asthma [12–15]. Sources of each of these cytokines are diverse in the asthmatic bronchial tree. Although T-lymphocytes produce IL-5, IL-3 and GM-CSF [10, 14, 15], epithelium [13, 16] and macrophages [14, 16] also produce GM-CSF and IL-3.

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There is controversy about cytokines enhancing the survival of eosinophil in asthmatic airways. In a study using BAL fluids after specific allergen challenges [17], IL-5 was mainly associated with BAL eosinophilia with a minor contribution by GM-CSF. However, a study using sputum obtained from symptomatic asthmatics showed a contradictory result [18]. Thus, eosinophil survival-enhancing activity (ESEA) in BAL fluids was compared between asthmatic patients and normal subjects and the ESEA was characterized to identify the main eosinophil-active cytokine in terms of the prolongation of eosinophil survival. In addition, the relationship between ESEA and proportions of activated T-lymphocytes of BAL cells, as a source of eosinophil-active cytokines, was analysed.

Materials and methods

Subjects

This study included 11 symptomatic patients and nine asymptomatic patients with asthma and eight normal control subjects. They were all nonsmokers. Age and sex were matched between the groups. All asthmatic subjects met the criteria of the American Thoracic Society [19] on the

basis of clinical symptoms and physical examination. Each patient showed an airway reversibility documented by an inhalant bronchodilator-induced improvement of >15% in forced expiratory volume in one second (FEV₁) and/or airway hyperreactivity measured by the histamine challenge test. The symptomatic patients had asthmatic symptoms from days to several weeks before BAL. The asymptomatic patients were previously diagnosed as having bronchial asthma, but were free from asthmatic symptoms during the 4 weeks before BAL.

Atopy was determined by the presence of a positive immediate skin response or specific immunoglobulin E (IgE) in serum to one or more allergens including dust mites, cat fur, dog fur, cockroaches and grass pollens. Six symptomatic patients, five asymptomatic patients and four control subjects showed atopy. All subjects showed no evidence of respiratory infections during the 4 weeks before the study. FEV₁, forced vital capacity (FVC) and peak expiratory flow rate (PEFR) were measured 2 days before BAL. The symptomatic patients had significantly lower values of FEV₁, FVC and PEFR than the asymptomatic patients and normal subjects. Airway reactivity was measured by the provocative concentration of histamine required to reduce FEV₁ by 20% of prechallenge value (PC₂₀). The mean value of PC₂₀ histamine was significantly lower in both the symptomatic and the asymptomatic patients with asthma than that of the normal subjects (table 1). This study was performed with the approval of the Ethics Committee of Soonchunhyang University Hospital and informed written consent was obtained from all study subjects.

Bronchoalveolar lavage and preparation of fluids

No patient with bronchial asthma had ever used inhaled or systemic steroids before the study. In symptomatic patients with asthma, BAL was carried out after clinical improvement by treatment with an *i.v.* infusion of aminophylline (5 mg·kg⁻¹·day⁻¹) and inhalation of aqueous β₂-agonist (salbutamol 6–10 mg·day⁻¹). Both aminophylline and salbutamol were stopped 12 h before BAL. BAL was performed with fiberoptic bronchoscopy (Olympus B2-10, Olympus, Tokyo, Japan) after premedication with atropine and diazepam and local anaesthesia with 20 mL of 2% lidocaine. Each subject inhaled 200 µg of salbutamol from a metered-dose inhaler before bronchoscopy. Lavage was performed by instilling four aliquots (50 mL each) of

sterile and warm saline at the right middle lobe. The lavage fluid was withdrawn immediately by gentle suction with negative pressure below 50 mmHg and collected in polyethylene tubes on ice. After filtering through a layer of gauze, the cell number was counted in a haemocytometer. BAL fluids were separated from cell pellets by centrifugation at 500×g for 5 min at 4°C. Cell pellets were brought to a concentration of 2×10⁶ cells·mL⁻¹ for slide preparation and flow cytometry. Cytochrome slides were air-dried, fixed in methanol and stained (Diff-Quik; American Scientific Products, Chicago, IL, USA). Five hundreds of leukocytes were enumerated for differential counts. The BAL fluids of symptomatic patients with more than 3% eosinophils in BAL cells were selected for measurement of ESEA. The lavage fluids were concentrated 100-fold using a Centricon-10 concentrator (Amicon Co., Beverly, MA, USA) with a molecular weight cut-off of 10,000 kDa. No complications were experienced in any subjects during the BAL procedures.

Purification of eosinophils

Eosinophils were isolated from the peripheral blood of atopic patients by the discontinuous Percoll gradients method [20]. In brief, heparinized venous blood was sedimented with 5% dextran in 0.9% NaCl at room temperature for 60 min to remove erythrocytes. A leukocyte-rich layer was aspirated, centrifuged at 300×g for 10 min, then washed twice in piperazine diethanesulphonic acid (PIPES) buffer (25 nM PIPES, 110 mM NaCl, 5 mM KCl, 40 mM NaOH and 5.4 mM glucose). Percoll and PIPES buffer were mixed to obtain solutions of the following density (in g·mL⁻¹): 1.100 (1.5 mL), 1.090 (3 mL), 1.085 (3 mL), and 1.080 (3 mL). The osmolarity of Percoll ranged from 280–310 mosmol·kg⁻¹ and the pH was 7.4. These were then layered at the indicated volumes in 140×10 mm polystyrene tubes. Cells were suspended in 2 mL of the Percoll solution (1.070 g·mL⁻¹), layered on the top of the gradients and centrifuged at 1,600×g for 30 min at 4°C. The fractions containing eosinophils were pooled and washed twice in RPMI-1640 medium. The eosinophil-enriched fractions were incubated with CD16 monoclonal antibody (Mab)-conjugated microbeads (Miltenyi Biotec, Belgisch-Gladbach, Germany) and a negative selection was conducted with a magnetic cell separator (MACS System; Becton-Dickinson, Mountain View, CA, USA) to remove the contaminating neutrophils [21]. The purity of eosinophils was >98%, as determined by light-microscopic examination of cytochrome slides prepared with Diff-Quik stain. Viability of eosinophils was determined by exclusion of propidium iodide (2 µg·mL⁻¹) with a flow cytometer (FACScan; Becton-Dickinson) [22]. The viability was >98% at the beginning of culture.

Measurement of eosinophil survival-enhancing activity in bronchoalveolar lavage fluids

ESEA was measured as described previously [8, 17, 18]. In brief, 1×10⁶ eosinophils were suspended in 1 mL of RPMI-1640 containing 1% human serum albumin (tissue culture medium (TCM)). Cell suspensions (150 µL) were mixed with 75 µL of the unconcentrated BAL fluids,

Table 1. – Profiles of the study populations

	NC	SBA	Asym BA
Age yrs*	35 (25–52)	34 (24–65)	33 (28–58)
Sex M/F	5/3	6/5	6/3
FEV ₁ % pred†	96.5±10.3	72.5±4.5#	88.5±4.8
FVC % pred†	98.5±9.7	76.7±8.1#	96.7±5.7
PEFR % pred†	91.4±7.9	69.0±4.6#	86.2±6.6
PC ₂₀ mg·mL ⁻¹ ‡	17.4±4.3	0.86±0.6§	1.43±0.7§
Atopy n	4	6	5

*: median (range); †: mean±SD; ‡: geometric mean±SD. NC: normal controls; SBA: symptomatic bronchial asthma; Asym BA: asymptomatic bronchial asthma; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; PEFR: peak expiratory flow rate; PC₂₀: provocative concentration of histamine causing a 20% fall in FEV₁. #: p<0.01 versus NC and Asym BA; §: p<0.01 versus NC.

the 100-fold concentrated BAL fluids (33-fold, final concentration), IL-3, IL-5, GM-CSF (Genzyme, Cambridge, MA, USA) and TCM in 96-well flat-bottomed tissue-culture plates, then incubated at 37°C in a 5% CO₂ humidified chamber. The viability of eosinophils was measured again at 72 h of culture as described above. Survival of eosinophils was calculated as the number of viable cells at 72 h of culture divided by that at the start of culture.

To characterize eosinophil-active cytokines in BAL fluids, the 100-fold concentrated BAL fluids were pretreated with MAb for 1 h at 4°C. The MAb included mouse anti-human GM-CSF (10 µg·mL⁻¹, final concentration), anti-human IL-3 (50 µg·mL⁻¹) and anti-human IL-5 (10 µg·mL⁻¹, Genzyme). Each MAb was specific for its respective cytokine and was not cross-reactive with other cytokines. Cell suspensions (150 µL) were mixed with 75 µL of the MAb-treated BAL fluids or TCM, then incubated at 37°C in a 5% CO₂ humidified chamber for 72 h. All experiments were carried out in triplicate. The inhibition percentage of ESEA by anti-cytokine MAb was determined by the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{ES (BAL)} - \text{ES (TCM)}) - (\text{ES (BAL plus anti-cytokine MAb)} - \text{ES (TCM)})}{\text{ES (BAL)} - \text{ES (TCM)}} \times 100$$

where ES is the percentage of eosinophil survival.

Expression of low-affinity interleukin-2 receptor on T-cell subsets of bronchoalveolar lavage cells

BAL cells were washed twice and suspended in Ca²⁺ and Mg²⁺-free phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.2% sodium azide. To enumerate the expression of low-affinity receptors for IL-2 (CD25) on T-lymphocyte subsets, dual immunofluorescence staining with MAbs was performed according to the standard method recommended by the Becton-Dickinson Monoclonal Center. Aliquots containing 1×10⁶ cells were incubated for 30 min at 4°C in the saturating concentrations of MAbs conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin. Each MAb for CD3 (Leu-2), CD4 (Leu-4) and CD8 (Leu-3) was a conjugated form with FITC. MAb for CD25 (anti-Tac) was a conjugated form with phycoerythrin (Becton-Dickinson). The surface immunofluorescence was analysed with a FACscan (Becton-Dickinson). The cells were excited with a 488-nm argon laser. The filter set to separate the two signals was recommended by the manufacturer for FITC and phycoerythrin. Each sample was measured up to 10,000 cells. The data were collected and analysed using the Consort 40 program (Becton-Dickinson).

Statistics

Differences between independent samples were compared using the nonparametric Kruskal-Wallis H-test for the continuous data. When found significant, the Mann-Whitney U-test was applied to compare the difference between two samples. The Wilcoxon signed rank test was applied to the changes of ESEA in the concentrated BAL

fluids by pretreatment with MAbs. The relationship between two parameters was studied using Spearman's rank correlation. The difference was considered significant when p-value <0.05. The results were expressed as means±SEM unless stated otherwise.

Results

Eosinophil survival-enhancing activity in bronchoalveolar lavage fluids

Survival of eosinophils decreased rapidly by 72 h of culture without eosinophil-active cytokines. IL-5, GM-CSF and IL-3 significantly increased the survival of eosinophils in a dose-dependent manner from 1 pg·mL⁻¹ to 10 ng·mL⁻¹ of each cytokine when compared with that by TCM alone. The order of potency to enhance survival was IL-5 > GM-CSF > IL-3 (at the same concentrations) (fig. 1). BAL fluids were concentrated because ESEA was not detected in the unconcentrated BAL fluids of seven symptomatic and five asymptomatic patients with asthma. To examine the change in ESEA of IL-3, IL-5 and GM-CSF by the concentrating procedure with the ultrafiltration membrane, the ESEA of each cytokine (1 ng·mL⁻¹ each) was compared before and after processing with a Centricon-10. The recovery rate of the ESEA for each cytokine was >90%.

The concentrated BAL fluids from eight of the 11 symptomatic patients enhanced the survival of eosinophils. The mean value of ESEA in symptomatic asthma was significantly higher than that of TCM (31.2±5.5 versus 8.1±0.5%, p<0.001). In contrast, the values of normal subjects (9.3±1.0%) and asymptomatic patients (10.4±0.9%) did not show an enhanced survival of eosinophils compared with TCM (p>0.05). The symptomatic patients had a significantly higher mean value of ESEA than that of asymptomatic patients (p<0.001) and that of normal controls (p<0.001) (fig. 2). The mean value of atopic patients was not significantly different from that of nonatopic patients in the symptomatic asthma group (30.8±8.7 versus 31.4±5.2%, p>0.05).

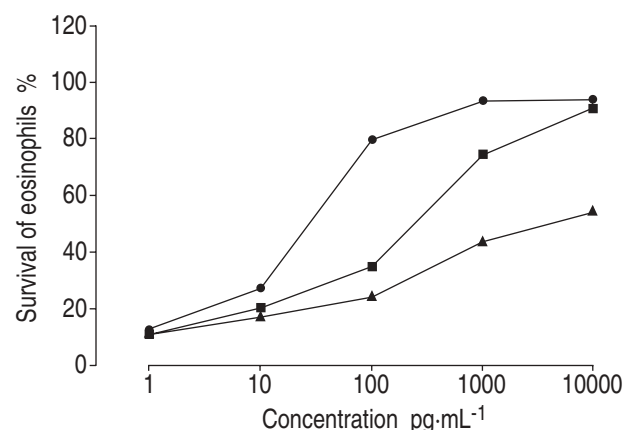


Fig. 1. – Eosinophil survival-enhancing activity of interleukin (IL)-5 (●), granulocyte macrophage colony-stimulating factor (GM-CSF; ■) and IL-3 (▲) *in vitro*. Eosinophil survival was measured at 72 h of culture with IL-5, GM-CSF and IL-3 (1 pg·mL⁻¹ to 10 ng·mL⁻¹). Each cytokine significantly increased the survival of eosinophils in a dose-dependent manner.

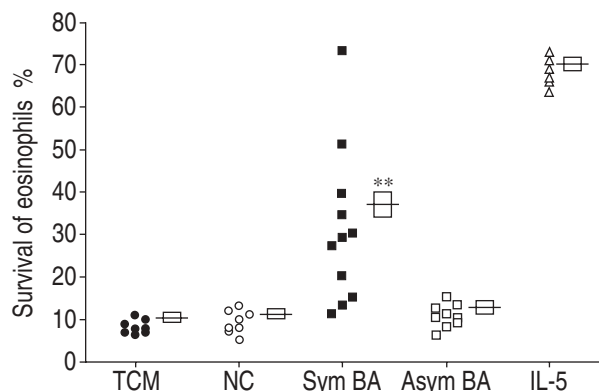


Fig. 2. – Eosinophil survival-enhancing activity in concentrated bronchoalveolar lavage (BAL) fluids from the study subjects. (Individuals and means \pm SEM.) The concentrated BAL fluids from the symptomatic bronchial asthma patients (Sym BA) significantly enhanced the survival of eosinophils when compared with tissue culture medium (TCM), those from the normal controls (NC) and the asymptomatic patients (Asym BA). Interleukin (IL)-5 (1 ng·mL⁻¹) was used as a positive control. **: $p < 0.001$ versus TCM, NC and Asym BA.

To examine the effect of BAL fluids on ESEA of IL-5, IL-3 and GM-CSF, the concentrated BAL fluids of a normal subject (ESEA: 11.2%), a symptomatic patient (ESEA: 51.2%) and an asymptomatic patient (ESEA: 13.2%) were mixed with IL-5, IL-3 and GM-CSF (1 ng·mL⁻¹), then the ESEA was compared with that observed with the same cytokine in TCM only (ESEA: 10.8%). The concentrated BAL fluid of the symptomatic patient significantly augmented the ESEA of IL-5, IL-3 and GM-CSF (fig. 3), but neither the BAL from a normal subject nor from an asymptomatic patient changed the ESEA of each cytokine (data not shown).

Identification of eosinophil-active cytokines in bronchoalveolar lavage fluids of the symptomatic patients with asthma

BAL fluids of six symptomatic patients with high ESEA (27–73%) were selected for the blocking study. Pretreat-

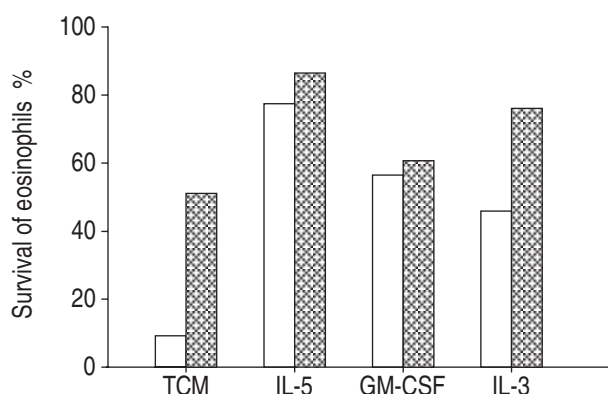


Fig. 3. – Effect of concentrated bronchoalveolar lavage (BAL) fluid on the eosinophil survival-enhancing activity (ESEA) of interleukin (IL)-5, IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF). A concentrated BAL fluid of symptomatic patients (ESEA: 51.2%) was mixed with IL-5, IL-3 and GM-CSF (1 ng·mL⁻¹), then the ESEA was compared with that observed with the same cytokine in tissue culture medium (TCM) only (ESEA: 10.8%). □: without BAL fluid; ▨: with BAL fluid. The concentrated BAL fluid significantly augmented the ESEA of IL-5, IL-3 and GM-CSF.

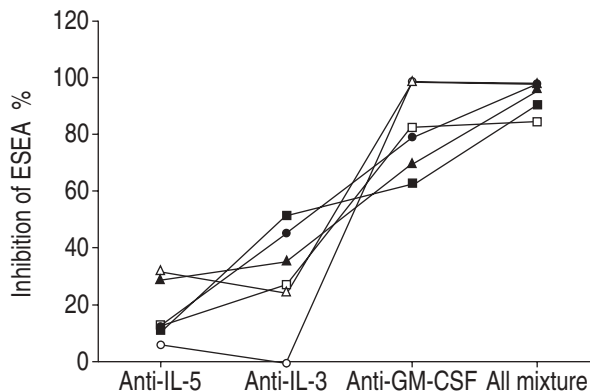


Fig. 4. – Inhibition of the eosinophil survival-enhancing activity (ESEA) in bronchoalveolar lavage (BAL) fluids from symptomatic patients by monoclonal antibodies to interleukin (IL)-5, IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF). Pretreatment with anti-GM-CSF completely neutralized the ESEA in two of six patients. The mean percentage of inhibition of ESEA was 83.3 \pm 7.8% by anti-GM-CSF, 31.6 \pm 7.7% by anti-IL-3 and 14.4 \pm 4.3% by anti-IL-5. A mixture of anti-IL-5, anti-IL-3 and anti-GM-CSF completely inhibited the ESEA from four of six cases. ○, □, △: atopic asthma; ●, ■, ▲: nonatopic asthma

ment with antibodies to IL-5, IL-3 or GM-CSF significantly reduced the ESEA in the concentrated BAL fluids (fig. 4). Pretreatment with anti-GM-CSF antibody completely neutralized the ESEA in two of six cases. The mean percentage of inhibition was 83.3 \pm 7.8% by anti-GM-CSF antibody, 31.6 \pm 7.7% by anti-IL-3 antibody and 14.4 \pm 4.3% by anti-IL-5 antibody. The inhibitory percentage by anti-GM-CSF was significantly higher than that of both anti-IL-5 antibody and anti-IL-3 antibody ($p < 0.05$). However, there was no significant difference between the inhibition of ESEA by anti-IL-3 antibody and that by anti-IL-5 antibody ($p > 0.05$). A mixture of antibodies for IL-5, IL-3 and GM-CSF totally inhibited the ESEA in four cases.

Cellular profiles of bronchoalveolar lavage cells and relationships with eosinophil survival-enhancing activity in the symptomatic patients with asthma

The symptomatic patients with asthma had greater percentages of eosinophils, neutrophils and lymphocytes among BAL cells compared with those of the normal subjects. The percentage of eosinophils was higher in the symptomatic patients than in the asymptomatic patients ($p < 0.01$) (table 2). In the symptomatic patients, ESEA correlated significantly with the percentage of eosinophils ($r = 0.82$, $p < 0.01$) of BAL cells, but not with that of neutrophils ($r = 0.22$, $p > 0.05$), lymphocytes ($r = -0.06$, $p > 0.05$) or macrophages ($r = -0.57$, $p > 0.05$). ESEA correlated significantly with the percentages of CD25(+)CD3 lymphocytes ($r = 0.78$, $p < 0.01$) and CD25(+)CD4 lymphocytes ($r = 0.69$, $p < 0.05$), but not with the percentages of either T-cell subsets or CD25(+)CD8 lymphocytes (table 3). The percentage of eosinophils correlated with the percentage of both CD25(+)CD3 lymphocytes ($r = 0.81$, $p < 0.01$) and CD25(+)CD4 lymphocytes ($r = 0.77$, $p < 0.05$). ESEA of the symptomatic patients with asthma did not correlate significantly with PEFR ($r = -0.14$, $p > 0.05$) FEV₁ ($r = -0.18$, $p > 0.05$) or PC20 ($r = -0.21$, $p > 0.05$).

Table 2. – Cellular profiles of bronchoalveolar lavage cells

	NC		SBA		Asym BA	
Study number	7		11		9	
Total number $\times 10^6$	19.3	(9.2–29.5)	24.5	(12.3–48.6)	19.2	(11.6–33.2)
Recovery %	50.2	(38.7–62.8)	46.3	(27.5–70.1)	49.0	(35.3–55.2)
Macrophages %	93.8	(89.2–98.5)	72.0	(65.1–89.9)**	90.6	(85.3–97.6)
Lymphocytes %	4.2	(2.0–11.2)	13.9	(4.4–43.9)#	5.3	(3.2–13.2)
Neutrophils %	0.9	(0.0–2.3)	2.3	(0.4–11.8)#	1.6	(0.1–3.3)
Eosinophils %	0.8	(0.0–1.5)	12.5	(4.4–50.4)+*	3.2	(0.6–6.8)#

Values are expressed as median (range). NC: normal controls; SBA: symptomatic bronchial asthma; Asym BA: asymptomatic bronchial asthma. **: $p < 0.01$ versus NC and Asym BA; #: $p < 0.01$; +: $p < 0.001$ versus NC; *: $p < 0.05$ versus Asym BA.

Discussion

This study confirmed that the BAL fluids of symptomatic patients with asthma significantly increased the survival of eosinophils *in vitro*. In the measurement of ESEA, concentration of the BAL fluids was necessary because ESEA was not detected in the unconcentrated BAL fluids. Concentration ratios vary among the studies measuring ESEA. OHNISHI *et al.* [17] observed ESEA in the 4-fold diluted BAL fluids, which were obtained at 48 h after segmental allergen challenges. In contrast, in a study of symptomatic patients with nocturnal asthma, JARIJOUR and BUSSE [23] did not find ESEA in the 10-fold concentrated BAL fluids. One explanation for the lack of ESEA in the study of JARIJOUR and BUSSE [23] is that the BAL fluids might not have been concentrated enough for the detection of ESEA. The other possibility is the presence of in-hibitory factors in BAL fluids. OHNISHI *et al.* [24] observed an inhibitory activity with a molecular weight $< 3,000$ kDa in BAL fluids. Lidocaine was suggested to be the inhibitor because of its ability to inhibit the survival of eosinophils *in vitro*. In the present study, Centricon-10 with a molecular weight cut-off of 10,000 kDa was used to concentrate the BAL fluids. Thus, lidocaine would be removed from the BAL fluids, and no inhibitory effect was observed in the concentrated BAL fluids on the ESEA of IL-3, IL-5 and GM-CSE. Another possibility is that a heavy exposure to allergens in cases of segmental allergen challenges might produce a greater amount of cytokines than the natural aggravation of asthma in the patients of this study.

It was demonstrated that GM-CSF was the major cytokine responsible for the ESEA in the BAL fluids of symptomatic patients. A similar neutralization method was used with monoclonal antibodies for IL-3, IL-5 and GM-CSF

as described in the other studies [17, 18, 23, 24]. However, OHNISHI *et al.* [17] reported that IL-5 was mainly associated with ESEA in BAL fluids of the late response following segmental bronchial provocation, with a small contribution from GM-CSF. The discrepancy between the results may originate from the different backgrounds of the study populations. Their study subjects suffered from allergic rhinitis without bronchial hyperreactivity, whereas symptomatic asthma was employed in this present study. In the studies employing patients with symptomatic asthma, OHNISHI *et al.* [24] observed that IL-5 was a predominant eosinophil-active cytokine in diluted BAL fluids from some of the patients. In contrast, ADACHI *et al.* [18] found that GM-CSF was primarily associated with ESEA, with a partial contribution from IL-5 in diluted sputum of symptomatic asthmatics, which was consistent with the present result. We cannot explain the causes of the different results between these studies.

Eosinophil-active cytokines are synthesized by a variety of cells in the bronchial tree. GM-CSF and IL-3 are produced by T-lymphocytes, epithelium [13, 16] and monocytes [25] in the bronchial mucosa. IL-5 is derived from T-cells and mast cells in the bronchial tree [12, 14]. Eosinophils also produce IL-5 and GM-CSF [26]. Bronchial myofibroblasts prolong eosinophil survival by tumour necrosis factor- α and IL-1-mediated synthesis of GM-CSF [27]. The ESEA correlated with the percentage of eosinophils of BAL cells, but not with those of other inflammatory cells. This suggests that the ESEA of symptomatic patients is specific for eosinophils. A significant correlation was also observed between the percentage of CD25(+)CD4 lymphocytes and the ESEA, which means that the activated T-lymphocytes might be a source of the ESEA. The activated T-helper cells are evidently increased in the peripheral circulation and the bronchial tree of symptomatic asthmatics, [15, 16, 28]. They generate eosinophil-active cytokines, such as IL-3, IL-5 and GM-CSF [14, 29]. In a study using the T-cell line of BAL cells [30], polyclonal CD4+ T-cell lines from asthmatics secreted significantly elevated quantities of both IL-5 and GM-CSF compared with lines from atopic and nonatopic controls. These data partially support the present result of a correlation between the percentage of CD25(+)CD4 lymphocytes and the ESEA in the BAL of symptomatic patients with asthma.

Investigation of local expression of eosinophil-active cytokines in the mucosa is also an important way in which to reveal the pathogenesis of asthma. In a study investigating α -IL-5 receptor mRNA expression in bronchial mucosa [31], EG2-positive eosinophils were the major cells positive for α -IL-5 receptor mRNA in asthmatics. This

Table 3. – Correlation coefficients between eosinophil survival-enhancing activity (ESEA) and bronchoalveolar lavage cells in symptomatic patients with bronchial asthma (n=11)

	ESEA		Eosinophils	
	r	p	r	p
Eosinophils	0.82	0.002		
Monocytes	-0.57	0.068	-0.81	0.002
Neutrophils	0.22	0.520	0.31	0.360
Lymphocytes	-0.06	0.896	0.16	0.642
CD3 lymphocytes	-0.13	0.719	0.19	0.591
CD25(+)CD3	0.78	0.008	0.81	0.005
CD4 lymphocytes	0.52	0.124	0.39	0.256
CD25(+)CD4	0.69	0.028	0.77	0.010
CD8 lymphocytes	-0.39	0.266	-0.09	0.802
CD25(+)CD8	0.21	0.266	0.27	0.454

provides strong evidence that IL-5 may play an important role in stimulating eosinophils in the mucosa of asthmatics via the specific α -subunit of its receptor. In a study by KOTSIMBOS *et al.* [32], the number of α -GM-CSF receptor mRNA-positive cells correlated with the number of CD68-positive macrophages in the lamina propria of patients with asthma, but not with the number of EG2-positive cells. However, a co-localization study revealed that 15% of α -GM-CSF receptor mRNA-positive cells were positive for major basic protein. This suggests that GM-CSF is partially responsible for eosinophil survival in the lamina propria. The localization of IL-5, GM-CSF and IL-3 in the bronchial mucosa of symptomatic asthmatics was investigated using an immunohistochemical stain [33]. The main site of IL-5 expression was on the mononuclear cells of the lamina propria, whereas IL-3 and GM-CSF were expressed mainly on the epithelium. The predominant expression of GM-CSF on the epithelium was reported by SOUSA *et al.* [13] using hue saturation intensity colour image analysis. They observed that the epithelial cells of asthmatic subjects were stained significantly more with anti-GM-CSF antibody than those from normal subjects. In the subepithelial connective tissue, there was some staining in leukocytes and blood vessels, but this was not usually as extensive as staining of the epithelium.

These data suggest that granulocyte macrophage colony-stimulating factor synthesized predominantly by the epithelium on the basement membrane may permeate into the bronchial lumen more easily than interleukin-5 synthesized in the lamina propria below the basement membrane. This may explain the result that granulocyte macrophage colony-stimulating factor was the main eosinophil survival-enhancing cytokine in the bronchoalveolar lavage fluids of symptomatic asthmatics, rather than interleukin-5. Interleukin-5 may have an influence on the survival of eosinophils, mainly in the lamina propria, and granulocyte macrophage colony-stimulating factor mainly in the epithelial layer and bronchial lumen. To explain the exact process of eosinophil infiltration into the airway of symptomatic asthmatics, a quantitative measurement of eosinophil-active cytokines at the level of individual cells in the asthmatic bronchial tree would be required.

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