IL-5 production by bronchoalveolar lavage and peripheral blood mononuclear cells in asthma and atopy

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IL-5 production by bronchoalveolar lavage and peripheral blood mononuclear cells in asthma and atopy. C. Tang, J.M. Rolland, C. Ward, B. Quan, E.H. Walters. ©ERS Journals Ltd 1997.

ABSTRACT: There is increasing evidence to suggest a key role for interleukin-5 (IL-5) in the regulation of airway eosinophilia in asthma. We compared the capacity for IL-5 production in atopic asthmatic, nonatopic asthmatic, atopic nonasthmatic and normal subjects, and evaluated the usefulness of peripheral blood cells for reflecting airway cell reactivity.

Bronchoalveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMC) from 12 atopic and 10 nonatopic asthmatics (without inhaled steroid therapy), 9 atopic nonasthmatics, and 10 normal controls were cultured with or without house dust mite (HDM, 10 µg·mL^{-1}) stimulation. CD4+ T-cell activation, IL-5 and interferon-γ (IFN-γ) production in the cultures were assessed.

Both for BAL and PBMC samples, atopic and nonatopic asthmatic subjects showed comparable spontaneous production of IL-5, which was significantly higher than that either for atopic nonasthmatics or normal controls ($p<0.05$ or $p<0.01$). There was a significant correlation between the percentage of eosinophils in BAL and spontaneous production of IL-5 by BAL cells in both asthmatic groups ($p<0.05$ or $p<0.01$). Both these parameters were also associated with asthmatic airway narrowing as denoted by a negative relationship with baseline forced expiratory volume in one second (FEV1) as percentage predicted ($p<0.05$ and $p<0.01$, respectively). In contrast, IFN-γ production by unstimulated BAL cells from normal controls was higher than that for BAL cell cultures of nonatopic asthmatic subjects ($p<0.05$). Following HDM stimulation, both atopic groups had comparable positive responses in terms of CD4+ T-cell activation, but there was relatively greater IL-5 production in asthmatic PBMC ($p<0.05$) and, in particular, BAL cell cultures ($p<0.01$).

These findings suggest that elevated IL-5 production is a common feature of BAL cells and PBMC in atopic and nonatopic asthma. In addition, atopic asthmatics show greater IL-5 production in response to specific allergen compared with atopic nonasthmatics, an effect most marked in BAL cells compared to PBMC. Hence, peripheral blood mononuclear cells can reflect cytokine immunoreactivity of airway cells, but lack local cellular interactions, which limits their usage in asthma research.
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Asthma is characterized by infiltration of the bronchial mucosa with activated T-cells and eosinophils [1–6], and has been referred to as chronic eosinophilic bronchitis [7, 8]. Eosinophils, through their release of basic proteins and lipid mediators, are strongly implicated in the genesis both of epithelial cell damage and bronchial hyperresponsiveness [7, 8].

There is increasing evidence that specialized populations of activated T-cells are involved in the initiation and regulation of eosinophilic airway inflammation *via* production of an array of cytokines [8]. Interleukin-5 (IL-5), granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) have pronounced effects *in vitro* on the prolongation of eosinophil survival, maturation, activation, and adhesion to vascular endothelium [9–12]. These cytokines may, therefore, play a critical role in the migration and activation of eosinophils in the airways *in vivo*. This hypothesis is supported by studies demonstrating an increased level of IL-5 in bronchoalveolar lavage (BAL) fluid both from atopic and nonatopic asthmatic subjects [13, 14]. A parallel study has also shown spontaneous release of IL-5, GM-CSF and IL-3 by activated peripheral blood T-cells from such subjects [15], suggesting that the production of these cytokines in the airways may be reflected by similar activity in peripheral blood cell populations.

Consistent with these observations are studies employing the techniques of *in situ* hybridization and immunocytochemistry, which have shown elevated percentages of cells positive for IL-5, GM-CSF, IL-3 and interleukin-4 (IL-4), but not interleukin-2 (IL-2) and interferon-γ (IFN)-γ, in peripheral blood, BAL and bronchial biopsies obtained from atopic asthmatic patients compared

with nonasthmatic controls. The predominant cells implicated in this cytokine production are CD4+ T-lymphocytes [16–18].

Of the eosinophil-activating cytokines, IL-5 is specific for eosinophils as opposed to other leucocytes [19], and was recently identified as the most important constituent promoting eosinophil survival and activity in the lung 24 h after antigen challenge [20, 21]. Moreover, another recent study showed that the percentages of peripheral blood CD4+ lymphocytes from asthmatic subjects expressing messenger ribonucleic acid (mRNA) encoding IL-5 but not GM-CSF or IL-3 correlated both with the number of eosinophils in the blood and the severity of clinical disease [17]. Taken together, these studies suggest that preferential production of eosinophil-activating cytokines, in particular IL-5, by activated CD4+ T-cells is a feature of the pathogenesis both of atopic and nonatopic asthma, and that such lymphocyte reactivity may not necessarily be limited to the end organ involved in the disease, *i.e.*, the airway.

On the other hand, studies in allergen-induced late-phase cutaneous and nasal reactions in allergic nonasthmatic subjects, and segmental airway allergen challenge studies coupled with BAL in allergic rhinitis patients have suggested that production of eosinophil-activating cytokines, including IL-5, is associated with atopic disease and is not specific to asthma [22–24]. Thus, the relevance of elevated IL-5 production to the pathogenesis of asthma remains to be confirmed. It is also not clear to what extent increased production of IL-5 by peripheral blood T-cells reflects airway cell reactivity.

To evaluate the ability of IL-5 production to distinguish asthma from atopy, and to investigate how well peripheral blood T-cell reactivity reflects events in the airways, we have analysed IL-5 secretion by unstimulated and allergen-stimulated BAL cells and peripheral blood mononuclear cells (PBMC) from atopic and nonatopic asthmatic subjects, compared with atopic and nonatopic nonasthmatic subjects.

Methods

Subjects

Recruitment. In previous bronchoscopic studies of asthmatics, we have found it extremely difficult to recruit meaningful numbers of nonatopic asthmatics due to the preponderance of atopic asthma in Australia. This study could not be completed without this group and, therefore, all subjects were recruited for this study at a collaborating centre with a more balanced profile of asthmatic patients and where it was possible to admit patients overnight after research procedures (Department of Respiratory Medicine, 202 Hospital, Shenyang, China). The majority of the practical work was also performed at this centre, with laboratory expertise, methods and reagents provided from Melbourne.

A total of 12 atopic asthmatic (AA), 10 nonatopic asthmatic (NAA), 9 atopic nonasthmatic (AN), and 10 normal control subjects (N) was studied (table 1). Asthmatic subjects had a history of periodic wheeze requiring intermittent inhaled β_2-agonist therapy, and documented reversible airway obstruction (20% improvement in forced expiratory volume in one second (FEV_1) in response to inhaled β_2-agonist). None had received orally-administered or inhaled corticosteroids in the 3 months preceding the study. Atopic nonasthmatic subjects had a history of allergic symptoms, such as allergic rhinitis (six subjects) or conjunctivitis (three subjects) but no chest tightness, wheeze or shortness of breath. They all showed normal baseline FEV_1. All atopic asthmatic and atopic nonasthmatic subjects showed positive skin-prick test reactions to house dust mite antigen (HDM) and to one or more other allergens. Nonatopic asthmatic and normal control subjects had negative skin tests to a panel of common aeroallergens. The normal control subjects had no history of asthma, rhinitis or bronchitis, and had normal baseline FEV_1.

All the subjects were nonsmokers and had been free from clinical, symptomatic respiratory infections for at least 6 weeks at the time of study.

The protocol was approved by the Scientific Research Committee, 202 Hospital (Shenyang, China). Each participant gave informed consent prior to entering the study.

Histamine inhalation tests

Histamine inhalation tests were performed before bronchoscopy according to a standardized method, adapted from the protocol described by COCKCROFT *et al.* [25]. Theophylline and inhaled or orally-administered β_2-agonists were withheld for at least 12 h before the tests. The FEV_1 was measured in triplicate with a wedge bellows spirometer (Vitalograph, UK), and the highest FEV_1 value was used as the baseline. Histamine was diluted with phosphate-buffered saline (PBS) to produce concentrations ranging 0.0625–32 mg·mL^{-1}. The aerosol was

Table 1. – Characteristics of the subjects studied

	Normal controls	Atopic nonasthmatics	Atopic asthmatics	Nonatopic asthmatics
Subjects n	10	9	12	10
Age yrs[‡]	30 (23–42)	35 (22–50)	35 (21–40)	39 (20–53)
Gender M/F	8/2	5/4	6/6	6/4
PC$_{20}$ mg·mL^{-1}[$]	ND	11.2 (3.8–32)	0.73 (0.25–4.2)***	0.76 (0.13–3.4)***
FEV_1 % pred[‡]	104 (89–125)	92 (79–123)	82 (66–99)++	84 (74–93)++

[‡]: median, and range in parenthesis; [$]: geometric mean, and range in parenthesis. M: male; F: female; PC$_{20}$: provocative concentration of histamine producing a 20% fall in FEV_1; ND: not determined; FEV_1: forced expiratory volume in one second; % pred: percentage of predicted value. ++: $p < 0.01$, compared with control group; *,***: $p < 0.05$, $p < 0.001$, compared with atopic nonasthmatic group.

generated using nebulizers containing 5 mL of solution and an airflow rate of 16 L·min^{-1}. The nebulizers delivered a reproducible gravimetric output of 0.28–0.3 mL·min^{-1}. Histamine was inhaled by tidal breathing for 0.5 min in doubling concentrations at 2 min intervals until a 20% decrease in FEV1, recorded in the last minute of the interval between doses, was observed or the highest histamine concentration was given.

Bronchoscopic procedure

Fibreoptic bronchoscopy was undertaken according to the guidelines of the American Thoracic Society [26]. All subjects were given nebulized albuterol (2.5 mg) and intramuscular atropine (0.5 mg) 15 min before the procedure. Thirty millilitres of venous blood was drawn into a heparinized container for later separation of mononuclear cells. Lignocaine 4% was used for local anaesthesia. Bronchoscopy was performed using an Olympus B1 IT10 flexible fibreoptic bronchoscope (Tokyo, Japan). Three 50 mL aliquots of sterile PBS at 37°C were individually instilled by gentle hand pressure into the medial segment of the right middle lobe, and were recovered by gentle suction. The BAL fluid was collected into polypropylene tubes and immediately placed on ice.

Cell processing and culture

BAL cells were washed twice with PBS. Cell counts and assessments of cell viability were performed with a haemocytometer and trypan blue exclusion staining, with results expressed as cells per millilitre of recovered lavage fluid. Cells were resuspended at 5×10^5 cells·mL^{-1} in RPMI-1640 medium (Sigma, USA) with 2 mM L-glutamine, 200 U·mL^{-1} penicillin, 200 µg·mL^{-1} streptomycin, and 5% heat-inactivated human AB positive serum. Aliquots (100 µL), of the cell suspension were then spun on to slides at 800 rpm for 2 min. After staining with May-Grünwald-Giemsa, differential cell counts were performed on two slides by counting 500 cells per slide, and a mean calculated.

The volume of BAL cell suspension was adjusted to give a final concentration of 2×10^6 cells·mL^{-1}. One millilitre of cell suspension was put into each well of a 24-well flat-bottomed culture plate (Costar, USA) with or without house dust mite antigen (HDM), 10 µg·mL^{-1} (Greer Laboratories, USA). Plates were incubated at 37°C in an air/5% CO_2 atmosphere for a time period (72 h) based on preliminary time course studies. Viability of the BAL cells assessed by trypan blue dye exclusion was generally >60% following culture and was similar between the four groups studied.

PBMC were separated from heparinized blood by density centrifugation on Ficoll-Paque (Pharmacia, Sweden). The cells were resuspended at a final concentration of 3×10^6 cells·mL^{-1} in RPMI-1640 medium with or without 10 µg·mL^{-1} HDM, and then incubated for a time (120 h) based on initial time course studies under the same conditions as described for BAL cells. Viability of the PBMC assessed by trypan blue dye exclusion was >80% following culture for all four study groups.

After incubation, BAL cells, PBMC and cell-free supernatants were obtained by centrifugation at 1500 rpm at 4°C for 10 min. Cells were resuspended at a concentration of 5×10^6 cells·mL^{-1} for flow cytometry. Cell-free supernatants were stored at -80°C until use.

Flow cytometry

Aliquots (50 µL) of BAL cells or PBMC suspension were incubated for 20 min at 4°C with aliquots (10 µL) of monoclonal antibodies against CD4 (conjugated with fluorescein isothiocyanate (FITC); Becton Dickinson, USA) and CD25 (conjugated with phycoerythrin (PE); Becton Dickinson, USA). FITC- and PE-conjugated immunoglobulins of matched isotypes (γ_1/γ_{2a} control; Becton Dickinson, USA) were used as negative controls. Cells were then washed twice with PBS containing 2% foetal calf serum (FCS).

Flow cytometric analysis was performed on the lymphocyte fraction as determined by characteristic forward and side scatter using laser excitation at 488 nm (Epics Profile II; USA). The number of immunofluorescence-positive cells was determined per 10,000 cells analysed.

Enzyme-linked immunosorbent assay (ELISA) for IL-5

Supernatants of BAL cell and PBMC cultures were assayed for IL-5 in duplicate by ELISA according to the procedure recommended by the manufacturer (PharMingen, USA). Briefly, microplates (Dynatech Laboratories, USA) were coated with purified rat monoclonal anti-IL-5 immunoglobulin G_1 (IgG$_1$) overnight at 4°C. Calibration and test samples were added to the plates for 4 h at room temperature, followed by biotinylated rat monoclonal anti-IL-5 immunoglobulin G_{2a} (IgG$_{2a}$) (PharMingen, USA) for 45 min at 37°C. Plates were rinsed six times. Binding of the second antibody was detected by stepwise incubation with avidin-peroxidase (Sigma, USA) and o-phenyldiamine/methanol (Sigma, USA). The reaction was terminated with 5 M H_2SO_4 and optical densities were read at 490 nm. The sensitivity of the assay was 25 pg·mL^{-1} and the coefficient of variation for the assay was 10%.

ELISA for interferon-γ (IFN-γ)

IFN-γ was measured in duplicate using a commercial ELISA kit (Medgenix, USA) according to the manufacturers instructions. The kit was suitable for the quantitative measurement of human natural and recombinant IFN-γ in cell culture medium, and was sensitive down to 100 pg·mL^{-1}.

Data analysis

After verifying normal distribution, the volumes of BAL fluid recovered were compared between groups for statistical significance using Student's t-test for unpaired observations. Data concerning the provocative concentration of histamine producing a 20% fall in FEV1 (PC20) were log transformed and analysed using Student's t-test. Spirometric data, BAL cell differential

counts, percentages of activated CD4 cells and cytokine levels were not normally distributed and were, therefore, expressed as medians with ranges, and tested for significance using the Mann-Whitney U-test for analyses between groups, and the Wilcoxon's matched signed-rank test for within-group comparisons. Correlations between the parameters of CD4 cell activation, cytokine production and eosinophil percentage in BAL were made using Spearman's rank correlation tests. When IL-5 values were below the limit of detection of the assay, a value of 0 was designated for statistical analysis. All analyses were performed using the Minitab statistical software package (CLE. COM; Birmingham, UK), and p-values of less than 0.05 were considered to be statistically significant.

Results

Characteristics of subject groups

Demographic details of the four subject groups studied are listed in table 1. PC20 histamine values were significantly lower in atopic and nonatopic asthmatic subjects than in atopic nonasthmatic subjects ($p < 0.001$). The test was not performed for normal controls. The two asthmatic subject groups also showed a significantly decreased mean prechallenge FEV1 (% pred) compared with normal subjects ($p < 0.01$) and atopic nonasthmatic subjects ($p < 0.05$).

BAL cell profiles are presented in table 2. Bronchoscopy was well-tolerated by the subjects and satisfactory recoveries of BAL fluid and cells were obtained in all cases. There were no significant differences between the four groups in the percentage recovery of BAL fluid and the total leucocyte concentration in the lavage. The percentages of macrophages and lymphocytes in the four groups were not significantly different. However, atopic and nonatopic asthmatic subjects had a significantly higher percentage of neutrophils ($p < 0.05$) than the normal subjects, and a significantly increased percentage of eosinophils than either normal subjects ($p < 0.01$) or atopic nonasthmatic subjects ($p < 0.05$).

Activation of CD4+ lymphocytes in PBMC and BAL cell cultures

The median (range) percentage of CD4+ CD25+ lymphocytes in unstimulated PBMC cultures did not differ

significantly between the four groups of subjects (AA 7.9 (5.0–11.7), NAA 7.4 (4.7–15.2), AN 7.0 (4.6–9.1), and N 6.1 (3.6–8.8) %), but it increased by a small but significant extent following HDM stimulation in both the atopic groups (AA to 8.6 (5.9–16.6) %; $p < 0.05$; and AN to 8.0 (5.5–12.9) %; $p < 0.01$) (fig. 1a).

BAL CD4+ lymphocytes from both asthmatic groups (atopic and nonatopic) were more activated in unstimulated cultures when compared with the nonasthmatic groups, as denoted by significantly higher median percentages of CD4+ CD25+ cells (AA 9.8 (8.0–13.1) and
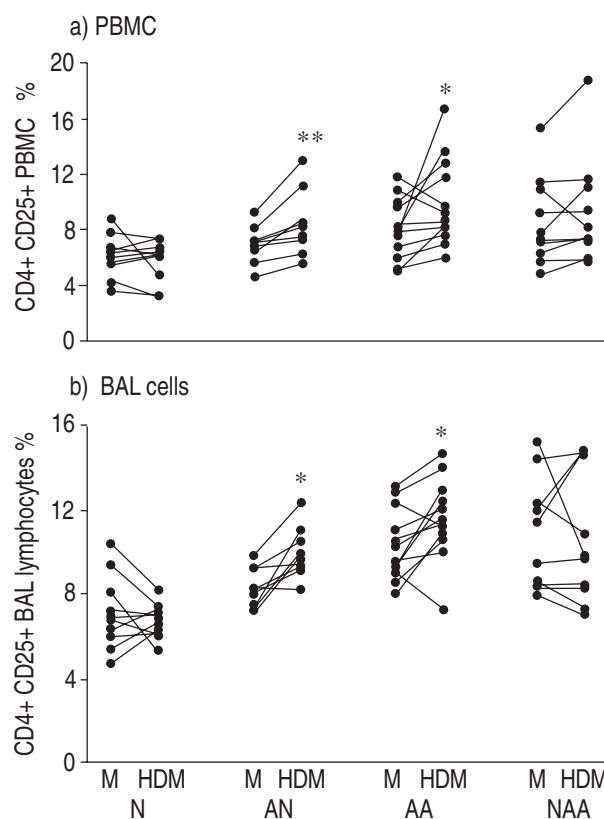

Fig. 1. – Percentages of CD4+ CD25+ lymphocytes in unstimulated (medium alone (M)) and HDM (10 µg·mL⁻¹)-stimulated cultures of: a) PBMC; and b) BAL cells from 10 normal control (N), 9 atopic nonasthmatic (AN), 12 atopic asthmatic (AA), and 10 nonatopic asthmatic (NAA) subjects. BAL: bronchoalveolar lavage; HDM: house dust mite allergen; PBMC: peripheral blood mononuclear cells. *: $p < 0.05$; **: $p < 0.001$, significance of increase with HDM stimulation.

Table 2. – Bronchoalveolar lavage (BAL) recovery and cell profiles

	Normal controls	Atopic nonasthmatics	Atopic asthmatics	Nonatopic asthmatics
BAL fluid recovery %	72 (67–80)	70 (66–81)	67 (63–77)	67 (53–79)
Total leucocytes ×10³ cells·mL⁻¹	90 (38–130)	70 (57–159)	61 (48–121)	73 (50–160)
Macrophages %	85 (76–88)	85 (76–89)	82 (72–90)	80 (71–88)
Lymphocytes %	13 (7–21)	15 (9–21)	14 (11–20)	17 (11–21)
Neutrophils %	1.6 (0.5–3.1)	1.9 (1.0–3.6)	2.9+ (1.1–8.5)	2.8+ (1.2–5.6)
Eosinophils %	0 (0–0.6)	0.4 (0–10)	2.4++* (0–5.5)	2.5++** (0.1–6.4)

Data are presented as medians, in range in parenthesis. +: $p < 0.05$, compared with normal control group; ++: $p < 0.01$, compared with normal control group; *: $p < 0.05$, compared with atopic nonasthmatic group; **: $p = 0.01$, compared with atopic nonasthmatic group.

NAA 10.4 (7.8–15.2) *versus* AN 8.3 (7.2–10.2) (p<0.05) and N 6.6 (4.7–10.4) % (p<0.01)) (fig. 1b). For PBMC cultures, HDM stimulation led to a significantly increased percentage of CD4+ CD25+ cells in BAL cell cultures in both the atopic groups (AA 11.5 (7.2–14.6) and AN 9.4 (8.2–12.3) %; p<0.05), but in neither of the nonatopic groups.

IL-5 levels in supernatants of PBMC and BAL cell cultures

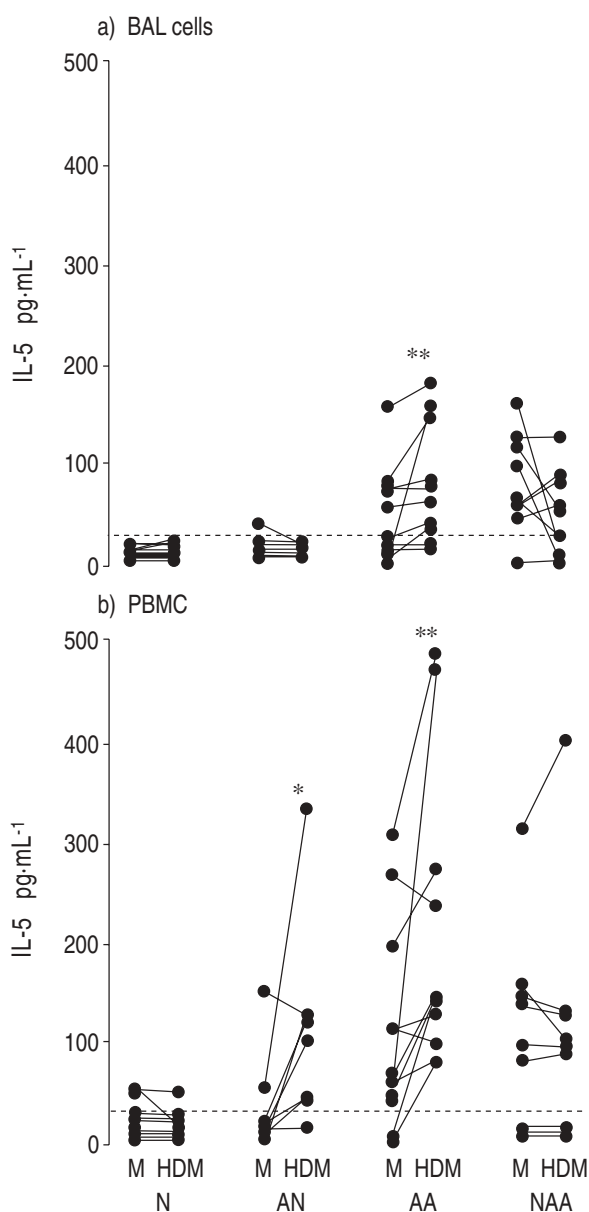There was a significantly higher IL-5 release by unstimulated PBMC from both the asthmatic groups (AA median (range) 74 (0–917.5) and NAA 80 (0–313.3) pg·mL^{-1}) when compared with the atopic nonasthmatic group (0 (0–151.1) pg·mL^{-1}; p<0.05) and normal control group (0 (0–52.6) pg·mL^{-1}, p≤0.01) (fig. 2).

A significant elevation in IL-5 release following HDM stimulation of PBMC was observed in the two atopic groups (AA to median 143 (79–488) pg·mL^{-1} (p<0.01); and AN to 102 (0–334) pg·mL^{-1} (p<0.05), but values were again higher for atopic asthmatics than those for atopic nonasthmatic subjects (p<0.05).

Consistent with the findings in unstimulated PBMC cultures, significantly higher levels of IL-5 were found in supernatants of unstimulated BAL cell cultures in both asthmatic groups (AA median 38 (0–158) and NAA 82 (0–161) pg·mL^{-1}) when compared with nonasthmatics (atopic and normal control subjects) (p<0.01), where levels were essentially undetectable. However, in contrast to PBMC, HDM stimulation of BAL cells led to a significant increase in IL-5 release only in the atopic asthmatics (to median 69 (0–181) pg·mL^{-1}; p<0.01).

IL-5 production by unstimulated but not HDM-stimulated BAL cells was significantly associated with the percentages of eosinophils recovered from BAL in both asthmatic groups (AA: r=0.617, p<0.05; NAA: r=0.842, p<0.01 (fig. 3a and b).

Significant correlations between the percentage of activated CD4+ CD25+ lymphocytes and IL-5 release were found only in HDM-stimulated PBMC cultures in the two atopic groups (AA: r=0.644, p<0.05; AN: r=0.845, p<0.01 (fig. 4). This relationship was not seen in the two nonatopic groups, or in HDM-stimulated

Fig. 2. – Interleukin-5 (IL-5) levels in supernatants of: a) BAL cell; b) PBMC unstimulated (medium alone (M)) and HDM (10 µg·mL^{-1})-stimulated cultures for 10 normal (N), 9 atopic nonasthmatic (AN), 12 atopic asthmatic (AA), and 10 nonatopic asthmatic (NAA) subjects. The points below the dotted line represent undetectable levels. For definitions see legend to figure 1. Significant increases with HDM stimulation. *: p<0.05; **: p<0.01, significance of increase with HDM stimulation.
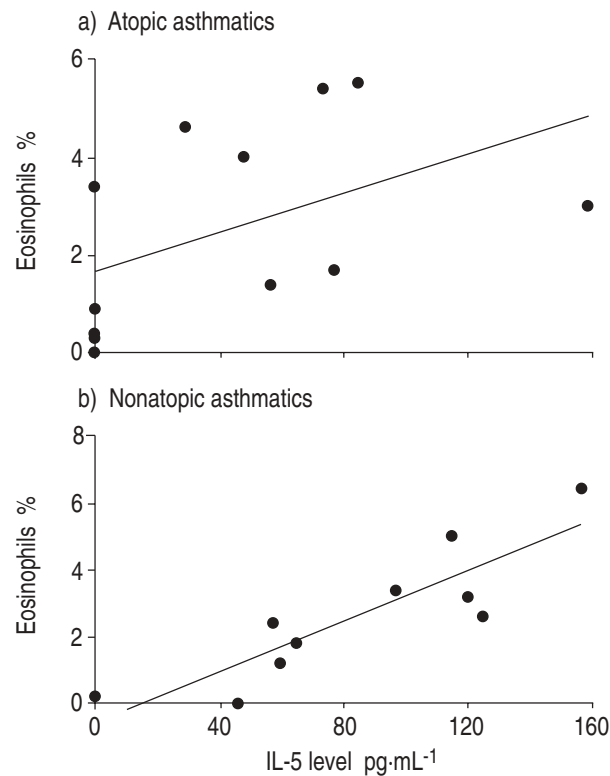

Fig. 3. – Relationship between interleukin-5 (IL-5) release from unstimulated bronchoalveolar lavage (BAL) cells and percentage of eosinophils recovered from BAL for: a) 12 atopic asthmatic subjects (r=0.617; p<0.05); and b) 10 nonatopic asthmatic subjects (r=0.842; p<0.01).

a) Atopic asthmatics
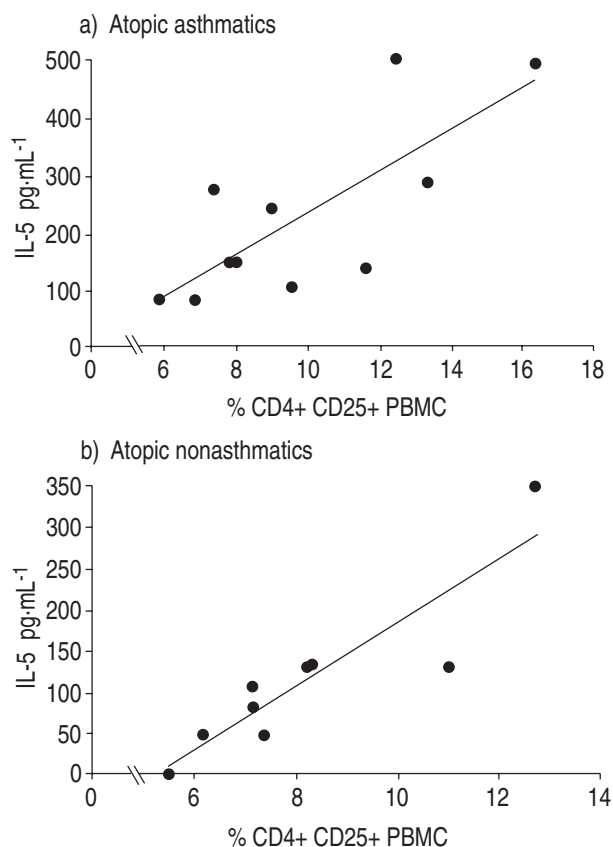

b) Atopic nonasthmatics

Fig. 4. – Relationship between percentage of CD4+ CD25+ lymphocytes and interleukin-5 (IL-5) production in HDM-stimulated PBMC cultures from: a) 12 atopic asthmatic subjects (r=0.644; p<0.05); and b) 9 atopic nonasthmatic subjects (r=0.845; p<0.01). For definitions see legend to figure 1.

BAL cell cultures in any of the four groups, nor in the cultures of unstimulated PBMC and BAL cells from the four groups.

IFN-γ levels in supernatants of BAL cell and PBMC cultures

Levels of IFN-γ in supernatants were measured as a marker of type 1 T-helper (TH1) reactivity. IFN-γ release by unstimulated PBMC and BAL cells from normal controls was found to be higher than that for the other

groups, but the difference was only significant for BAL cells when compared with nonatopic asthmatic subjects (p<0.05). HDM stimulation of PBMC and BAL cell cultures did not induce significant increases in IFN-γ production in any of the four groups (table 3).

No relationship was found between IL-5 production and IFN-γ production for individual BAL cell and PBMC cultures for any of the study groups.

Correlation of asthmatic airway narrowing with percentage of BAL eosinophils and IL-5 production

There was a significant negative association between baseline FEV1 % pred and percentage of BAL eosinophils both in atopic asthmatics (r=-0.564; p<0.05) and nonatopic asthmatics (r=-0.802; p<0.01). The same relationship was also found for FEV1 % pred and spontaneous production of IL-5 (but not IFN-γ) by BAL cells, but this was significant only in nonatopic asthmatics (r=-0.827; p<0.01) and not in atopic asthmatics (r=-0.486; p=0.09). Spontaneous production of IL-5 by PBMC did not relate to FEV1 % pred in either asthmatic group. However, patients with an FEV1 of less than 80% pred did show elevated levels of PBMC IL-5 production (median 156 (45–313) pg·mL^{-1}, n=7) compared with subjects with an FEV1 greater than 80% pred (median 78 (0–267) pg·mL^{-1}, n=15; p=0.05).

The relationships between PC20 histamine and percentage of BAL eosinophils or IL-5 production by unstimulated cells in the two asthmatic groups were not statistically significant.

Discussion

In this study, an elevated spontaneous IL-5 production was demonstrated both by BAL cells and PBMC *in vitro* from atopic and nonatopic asthmatic subjects compared to that from nonasthmatic subjects (including atopic and nonatopic). In addition, there was a significant correlation between the spontaneous IL-5 production by BAL cells and the percentage of eosinophils in BAL for both the asthmatic groups. Furthermore, allergen-specific responses were demonstrated, in terms of

Table 3. – Interferon-γ levels in supernatants of BAL cells and PBMC with or without HDM stimulation

	Interferon-γ level in supernatants pg·mL^{-1}			
	Normal controls	Atopic nonasthmatics	Atopic asthmatics	Nonatopic asthmatics
BAL cells				
Medium alone	605 (444–1210)	494 (370–975)	389 (185–864)	376* (124–1482)
HDM (10 µg·mL^{-1})	542 (420–1210)	605 (259–1160)	534 (124–878)	497 (171–1580)
PBMC				
Medium alone	769 (487–1712)	650 (238–950)	562 (50–>2000)	556 (62–1675)
HDM (10 µg·mL^{-1})	710 (482–>2000)	642 (444–963)	969 (234–1667)	599 (210–1346)

Data are presented as median, and range in parenthesis. BAL: bronchoalveolar lavage; PBMC: peripheral blood mononuclear cells; HDM: house dust mite antigen. *: p<0.05 compared with normal control group.

activation of CD4+ lymphocytes and cytokine production, in cultures of BAL cells and PBMC from atopic subjects with and without asthma. HDM stimulation led to an upregulation of CD4+ lymphocyte activation, as denoted by CD25 expression in BAL cell and PBMC cultures, and a hyperproduction of IL-5 by PBMC in both the atopic groups. In contrast, an allergen-induced increase in IL-5 production by BAL cells occurred only in atopic asthmatic subjects. These findings suggest that local release of IL-5 in the airway may be responsible for the characteristic airway eosinophilia both of atopic and nonatopic asthma. Furthermore, the quantitative difference in IL-5 production by airway cells that was demonstrated may be crucial for the presence or absence of overt clinical asthma in atopic subjects.

The increased capacity of airway lymphocytes to produce IL-5 both in atopic and nonatopic asthmatic subjects, compared to that in normal healthy controls, has been documented previously [14, 15]. We have confirmed these findings and extended this comparison to atopic nonasthmatics. Similar levels of IL-5 were released by unstimulated BAL cells *in vitro* in both asthmatic groups (atopic and nonatopic). In contrast, BAL cells from atopic nonasthmatic and normal control subjects showed essentially no spontaneous IL-5 production. We believe that the negative result for atopic nonasthmatics is important because it indicates that the capacity of BAL cells to produce IL-5 relates to asthma *per se* rather than atopy in general.

Compatible with this, a significant upregulation of CD4+ lymphocyte activation in unstimulated BAL cell cultures was shown in both the asthmatic groups compared with that in the nonasthmatic groups. Our data possibly indicate ongoing chronic activation *in vivo* of the airway lymphocytes both from atopic and nonatopic asthmatic subjects. These individuals may be in the process of being exposed to an extrinsic antigen (such as HDM) in the case of atopic asthmatic subjects, and to intrinsic stimulation (or an as yet unidentified antigen) in nonatopic asthmatic subjects. This demonstration of chronic activation of local CD4+ lymphocytes in asthmatic subjects confirms other studies showing elevation of activated CD4+ lymphocytes in BAL and bronchial biopsies obtained from subjects with symptomatic but stable asthma [15, 27].

Recent studies have indicated that IL-5 is likely to be predominantly produced by CD4+ lymphocytes in asthmatic subjects [17, 28]. Our study was limited, in that we did not define the cell source of the cytokine. The correlations between percentage of CD4+ CD25+ lymphocytes and production of IL-5 in BAL cell cultures were weak. There are many potential reasons for this observation. Little is currently known about the relationship between cell activation, cytokine mRNA transcription and cytokine synthesis/secretion, although this is likely to be complex [17]. In addition, in this study, BAL cell cultures probably contained cell types other than T-cells, capable of either producing IL-5 or modulating its production and release, and thus potentially confounding any possible relationships. The capacities of mast cells and eosinophils to secrete IL-5 have recently been demonstrated [29–31], and expression of IL-5 mRNA by these cells has been shown in BAL and bronchial biopsies from asthmatic but not normal control subjects [32]. Thus, study at a single cell level would be necessary to further identify the relationship between T-cell activation and cytokine production in BAL cell cultures. Nevertheless, the similar trends toward upregulation of CD4+ lymphocyte activation and IL-5 production in unstimulated BAL cell cultures in both the asthmatic groups suggested a functional relationship between the two biological events.

In contrast to the findings for HDM-stimulated BAL cell cultures in atopic asthmatic subjects, expression of CD4+ lymphocyte activation markers and production of IL-5 in the same cultures were not concordant for atopic nonasthmatic subjects. This again emphasizes the lack of direct linkage between cell activation and IL-5 production, and possibly indicates a suppression of IL-5 production by the allergen-stimulated CD4+ lymphocytes in BAL in the latter individuals. Although OHNISHI and co-workers [20, 21] have reported increased IL-5 levels in BAL *in vivo* after airway segmental allergen challenge of atopic subjects without asthma, it is not clear whether there was a quantitative difference in allergen-induced secretion of IL-5 in the airways between atopic asthmatic and atopic nonasthmatic subjects in these studies. Our study clearly demonstrated a dichotomy *in vitro* between the two atopic groups, though the sensitivity of the IL-5 assay in this study was limited at a concentration of 25 pg·mL^{-1}, which may have failed to show a small elevation in atopic nonasthmatics.

The low secretion of IL-5 by the allergen-stimulated BAL cells from atopic nonasthmatic subjects may provide a mechanistic explanation for a significantly lower percentage of BAL eosinophils in this situation compared with the levels in atopic and nonatopic asthmatic subjects. Although airway eosinophilia in atopic nonasthmatic subjects has been described by others [2, 33] at an intermediate level both for cell number and activation between atopic asthmatics and normal controls, this gradation was not apparent in the present study. It seems likely that the extent of expression of the asthmatic state is related to the relative local inflammatory change, in turn influenced by the relative quantity of IL-5 secreted in the airways. This point was further supported by the significant negative relationships between airway constriction and percentage of BAL eosinophils or spontaneous IL-5 production by BAL cells in both the asthmatic groups.

The discrepancy in IL-5 production by HDM-stimulated BAL cells *in vitro* between the two atopic groups is noteworthy but difficult to explain. This cannot be attributed to a difference in lymphocyte reactivity to the allergen, at least in terms of increased CD25 expression on CD4+ lymphocytes. Rather, the difference may reflect differential regulatory effects on T-cell responses to the allergen exerted by other cells, perhaps alveolar macrophages in particular, present in the BAL cell cultures. This might explain why there was a relationship between cell activation with HDM stimulation and IL-5 production with PBMC but not BAL cells. It has been proposed that resident "pulmonary alveolar" macrophages play an important role in the maintenance of local immunological homeostasis, by collectively restricting the local induction of T-cell responses to antigenic challenges in normal healthy individuals [34], whereas alveolar macrophages can and do facilitate T-cell allergen-specific

responses in the lungs of asthmatic subjects [35]. Thus, a number of different factors in the T-cell microenvironment may determine the profile of its cytokine production [36]. The relationship of alveolar macrophage functional change to the specific pattern of cytokine release from allergen-specific CD4+ lymphocytes in atopic asthma, remains to be determined.

In the present study, median baseline levels of IL-5 in PBMC cultures were significantly higher in asthmatics than in nonasthmatics (including atopic and nonatopic), as we have reported for a previous study [37]. Thus, in terms of spontaneous IL-5 production, T-lymphocytes in the peripheral blood closely resembled those in the airways. Recent studies in asthmatic subjects demonstrated that IL-5 production by peripheral blood T-lymphocytes was significantly correlated with disease severity and was markedly reduced following oral glucocorticoid therapy [16, 17]. Overall, this suggests that T-lymphocyte cytokine production in the bronchial mucosa of asthmatic subjects is reflected by that in the peripheral blood. However, our current study would also suggest that the significance of PBMC cytokine responses to allergen in the investigation of the pathogenesis of atopic asthma may be limited by the absence of modulating cellular interactions existing in the lung.

In contrast to IL-5 production, a relatively higher IFN-γ secretion by unstimulated BAL cells from normal control subjects was observed compared with the other three groups of subjects. In addition, no significant increase in IFN-γ production was induced by HDM stimulation of BAL cells or PBMC in vitro in either of the atopic groups. A previous study has shown decreased expression of IFN-γ mRNA in BAL cells in atopic asthmatic subjects compared with normal controls, and a significant increase in the expression of IFN-γ mRNA in asthmatic patients after treatment with prednisone [32, 38]. It has been shown that the type 2 T-helper (Th2) cytokine IL-4 inhibits synthesis of the Th1 cytokine IFN-γ and induces immunoglobulin E (IgE) synthesis in human lymphocyte cultures [39]. Thus, reduction in IFN-γ production is considered to be a feature of atopy. It is, therefore, difficult to explain the significance of our finding that a decreased IFN-γ production compared with normals was most marked in the nonatopic asthmatic subjects. Previous studies have been contradictory: one showed elevated production of this cytokine by T-cells in BAL from nonatopic asthmatics [14], whereas another study suggested normal production of IFN-γ in these circumstances [15].

In summary, we have shown significantly increased spontaneous IL-5 production in vitro by BAL cells and PBMC from atopic and nonatopic asthmatic subjects compared with normals. In addition, IL-5 levels were significantly related to the percentages of eosinophils in BAL in both types of asthmatic subjects. In vitro allergen-induced CD4+ lymphocyte activation responses in PBMC reflected those of BAL cells in both atopic subject groups, but the extent of allergen-induced IL-5 production by BAL cells and PBMC was significantly different in atopic nonasthmatic subjects. These findings highlight an important potential role for IL-5 in the pathogenesis of asthma, and suggest that the difference in allergen-induced IL-5 production in the airways between atopic asthmatic and atopic nonasthmatic subjects

is an important determinant of the degree of airway eosinophilia and, hence, of clinical disease in atopic subjects. PBMC can reflect cytokine immunoreactivity in the airways of asthmatics, but PBMC responses to allergen in vitro may be confounded because of lack of the local immunoregulatory mechanisms present in the lung.

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