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Cytokine Profiles of Stimulated Blood Lymphocytes in Asthmatic and Healthy Adolescents Across the School Year

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ABSTRACT

T cell cytokines play an important role in mediating airway inflammation in asthma. The predominance of a Th2 cytokine profile, particularly interleukin (IL)-4 and IL-5, is associated with the pathogenesis and course of asthma. The aim of this study was to test the hypothesis that a stressful life event alters the pattern of cytokine release in asthmatic individuals. Thirteen healthy controls and 21 asthmatic adolescents gave blood samples three times over a semester: midsemester, during the week of final examinations, and 2-3 weeks after examinations. Interferon- γ (IFN- γ), IL-2, IL-4, and IL-5 were measured from supernatants of cells stimulated with PHA/PMA for 24 h. Cells from asthmatic subjects released significantly more IL-5 during the examination and postexamination periods, whereas cells from healthy controls released significantly more IL-2 during the midsemester and examination periods, thereby indicating a bias for a Th2-like pattern in asthmatics and a Th1-like pattern in healthy controls. IL-4 and IL-5 production showed a marked decrease during and after examinations in healthy controls, whereas this decline was absent in asthmatics. The ratios of IFN- γ :IL-4 and IFN- γ :IL-5 also revealed significant changes in the profile of cytokine release across the semester. These results indicate differential cytokine responses in asthmatics that may become pronounced during periods of cellular activation.

INTRODUCTION

CHRONIC AIRWAY INFLAMMATION is a characteristic feature of asthma, and it has been proposed that activated T helper cells (Th) and their products, cytokines, play a central role in the regulation of airway inflammation. Two functional subsets of Th cells have been identified in the immune system: Th1 cells predominantly secrete interleukin-2 (IL-2) and interferon- γ (IFN- γ) but little IL-4 and IL-5 and facilitate cell-mediated immunity. Th2 cells, in contrast, produce IL-4, IL-5, IL-6, and IL-10 predominantly but little IL-2 and IFN- γ and participate in immediate hypersensitivity and allergic reactions.⁽¹⁻³⁾ Evidence suggests that the pathogenesis of asthma is associated with a predominance of Th2-type cytokine production, and these cytokines may be critical signals initiating and orchestrating the complex cellular events in airway inflammation and hyperresponsiveness.^(4,5)

IL-4 and IL-5 are two particularly important cytokines im-

plicated in the pathogenesis of asthma. IL-4 is a B cell-stimulating and differentiating factor and an early signal for isotype switching of B cells to produce immunoglobulin E (IgE).^(6,7) Induced production of IgE antibody is a fundamental feature of allergic asthma. The major functions of IL-5 include the differentiation, maturation, and activation of eosinophils, as well as the enhancement of eosinophil viability.⁽⁸⁾

In allergic asthmatics, peripheral blood (PB) cells were found to produce significantly more IL-4 and IL-5 than cells from normal controls.⁽⁹⁾ Similarly, PB cell cultures from atopic children released higher levels of IL-4 but not IFN- γ .⁽¹⁰⁾ It was demonstrated further that asthmatic individuals had higher percentages of circulating T cells expressing IL-4 and IL-5 mRNA, a cellular profile that was decreased with improved lung function following glucocorticoid treatment.⁽¹¹⁾

Stress has been found to alter Th1 and Th2 cytokine production.⁽¹²⁻¹⁵⁾ Stimulated PB cell cultures showed that academic examination stress induced significant changes in the pro-

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duction of IL-2 and IFN- γ . However, the effects of stress on the levels of IL-4 and IL-5 have not been tested. In view of long-standing speculation that stress exacerbates asthma symptoms,^(16,17) changes in cytokine production are a potential mechanism modulating the relationship between stress and asthma. The goal of this study, therefore, was to test the hypothesis that a stressful life event alters the pattern of cytokine release in asthmatic adolescents. Th1 (IL-2 and IFN- γ) and Th2 cytokines (IL-4 and IL-5) were investigated in relation to final examination stress, comparing the levels and responsiveness to this stressful life event between healthy controls and asthmatic individuals. In addition, examination-induced changes in eosinophils, IgE, and peak expiratory flow rates were evaluated.

SUBJECTS AND METHODS

Thirty-four students were recruited from two local high schools in Madison, WI. The study protocol was approved by the school district and Institutional Human Subjects Committee, and consent forms were obtained from each student and a parent. As can be seen in Table 1, subjects were divided into healthy control, with no known health problems, and asthma groups I and II, based on medications prescribed. The validity of this subdivision was also supported by their IgE and blood eosinophil levels (see Results). Most asthmatic students typically used 2 puffs of β -agonist or steroid inhalants or both twice a day or as needed. Ninety-four percent of the subjects were Caucasian ($n = 32$), and there was 1 African American and 1 Asian American. No significant differences were noted in age, racial distribution, or gender among the three groups. Between the two asthma groups, however, there were more female subjects in the Asthma I group than in the Asthma II group.

Procedures

Blood samples were collected three times across the fall semester of the school year: (1) midsemester in early December (baseline), (2) during final examination week in mid-January (Exam), and (3) again 2–3 weeks after final examinations (Post-exam). In each period, 5 ml of blood was collected into an EDTA-containing vacutainer, and 10–20 ml of blood was collected into a heparinized vacutainer via routine venipuncture between 7:30 and 8:45 AM. EDTA-treated samples were sent to a local laboratory for complete blood count with differentials. Heparinized samples were processed for cytokine analyses.

Heparinized blood was layered onto Ficoll-Hypaque (1.077 sg, Sigma) and centrifuged at 450g for 30 min at ambient temperature. Mononuclear cells (MNC) at the interface between plasma and Ficoll were carefully harvested and washed twice with sterile phosphate-buffered saline (PBS without Ca^{2+} , Mg^{2+} , pH 7.4, Gibco) and centrifuged at 450g for 10 min. The cells were then resuspended with complete medium (RPMI 1640 supplemented with HEPES 25 mM, L-glutamine 2 mM, gentamicin 50 $\mu\text{g}/\text{ml}$, and 10% heat-inactivated fetal bovine serum, FBS) to give a final cell concentration of $2 \times 10^6/\text{ml}$.

Cytokine induction and analyses

Using 24-well flat-bottom cell culture plates, 1×10^6 MNC in 500 μl complete RPMI medium were cultured for 24 h at 37°C with 5% CO_2 in the presence of phytohemagglutinin (PHA, 5 $\mu\text{g}/\text{ml}$) and phorbol myristate acetate (PMA, 10 ng/ml). The PHA and PMA cocktail was found to induce maximal cytokine production from T lymphocytes.⁽¹⁸⁾ Culture supernatants were then collected and stored in aliquots at -70°C until assayed.

IFN- γ , IL-2, IL-4, and IL-5 levels were determined by a standard, two-step sandwich, enzyme-linked immunosorbent assay (ELISA). Briefly, a microtiter plate was coated with a cytokine-specific monoclonal antibody (mouse antihuman IFN- γ , Endogen; mouse antihuman IL-2, R & D Systems; and mouse antihuman IL-4 and rat antimouse IL-5, PharMingen). Sample supernatants were added to the plate, followed by the addition of a biotinylated antibody directed against the specific cytokine (polyclonal goat antihuman IFN- γ , R & D Systems; polyclonal rabbit antihuman IL-2, PharMingen; and monoclonal rat antihuman IL-4 and IL-5, PharMingen). Biotinylation of the polyclonal antihuman IFN- γ and IL-2 was performed according to manufacturer's instructions using an N-hydroxysuccinimide ester with a long chain sidearm (NHS-LC biotinylation kit, Pierce, IL). The assay was developed with a streptavidin horseradish peroxidase homopolymer and the substrate, trimethylbenzidine (TMB). Cytokine levels were quantitated by comparison to a standard curve generated from known concentrations of recombinant cytokine. The sensitivity for IL-2, IL-4, IL-5, and IFN- γ was less than 25, 3, 3, and 100 pg/ml, respectively.

Blood analyses

A complete blood count with leukocyte differentials was determined at a local medical laboratory (General Medical Laboratories, Madison, WI). Plasma levels of IgE were measured at the local hospital laboratory.

TABLE 1. SUBJECT CHARACTERISTICS

	Healthy Control	Asthma I	Asthma II
<i>n</i>	13	11	10
Age (years)	15.8 \pm 0.6 ^a	16.0 \pm 0.6	16.2 \pm 0.8
Gender (F/M)	9/4	7/4	3/7
Asthma medication	None	Nonsteroidal bronchodilator inhalant	Steroid and nonsteroidal inhalant
Asthma duration (year)	0	7.9 \pm 4.7	9.7 \pm 5.2

^aMean \pm SD.

Peak expiratory flow rate (PEFR)

Peak expiratory flow rate (PEFR) was measured in asthmatic subjects only. Asthmatic subjects were provided with an Assess Peak Flow Meter (HealthScan, Cedar Grove, NJ) and asked to record peak flow readings daily for 2 weeks in each period. Subjects were instructed to perform three peak flows at bedtime before taking asthma medications and to record the highest value in the diary. Before this diary assignment, all subjects were trained on how to use the flow meter correctly until they demonstrated proficiency. The Assess Peak Flow Meter was tested to be highly accurate and reproducible, with an average flow error <0.25%.⁽¹⁹⁾

Statistical analysis

Data were analyzed using SPSS statistical software. Repeated measures analysis of variance (ANOVA) was employed to analyze the significance of overall group differences, changes in cytokine production, and cell responses across the three time points (Baseline, Exam, and Postexam), and group \times time interactions. When appropriate, one-way ANOVA was used to determine group differences at each time, with specific contrasts between healthy control and asthmatic groups. In addition, correlational analyses were performed using Pearson product-moment correlations.

RESULTS*Cytokine and blood profiles of the groups*

Cytokine Profile. The levels of cytokine production, as well as the ratios of Th1/Th2 cytokines, were examined in asthmatic and healthy control subjects. As can be seen in Table 2, there

were significant differences in the levels of IL-2 and IL-5 between the healthy control and asthma groups. During the Baseline period, cells from healthy controls produced significantly more IL-2 than did cells from asthmatics, particularly in comparison with the asthma II group: $F(2,31) = 3.49, p = 0.04$ among the three groups; $t(31) = 2.34, p = 0.026$ between the healthy control and combined asthma groups. In contrast, the production of IL-5 was significantly higher in asthmatic subjects during the Postexam period, when both asthma groups had more than threefold higher levels of IL-5 than control subjects: $F(2,23) = 4.72, p < 0.02$ among the three groups; $t(23) = 3.07, p = 0.005$ between the healthy control and combined asthma groups. A similar pattern of significant differences was found in the IL-2 and IL-5 responses during the Exam period between healthy control and asthma groups (Table 2). IL-4 and IFN- γ production, however, was not different among the three groups. In addition, the overall level of IL-4 released from the cells was considerably lower than the levels of the other cytokines.

Because of the purported role of Th2 cytokines in the pathogenesis of asthma, we examined whether there were differences between healthy and asthmatic subjects in the ratios of Th1 vs. Th2 cytokines. In this regard, the levels of IL-2 and IFN- γ were examined with respect to IL-4 and IL-5. The ratio data were transformed by base 10 logarithm to generate a normal distribution of the data. The IL-2:IL-5 ratio provided the greatest distinction among the three groups. Repeated measures ANOVA indicated a significant group difference, $F(2,23) = 5.07, p < 0.02$.

As illustrated in Table 3, specific contrasts between healthy control and asthma groups showed that healthy controls had significantly higher ratios of IL-2:IL-5 than asthmatic subjects at all time points. Similarly, the ratios of IFN- γ :IL-5 also were significantly higher in healthy controls than in asthmatics during the Exam and Postexam periods. IL-2 and IFN- γ ratios with respect to IL-4 levels, however, did not differ between groups.

TABLE 2. CYTOKINE LEVELS (PG/ML) FROM SUPERNATANT FOLLOWING 1-DAY CULTURE OF CELLS WITH PHA/PMA COCKTAIL

	Healthy (n = 13)	Asthma I (n = 11)	Asthma II, (n = 10)	Contrast (healthy vs. asthma) p
IL-2				
Baseline	13922 \pm 1547 ^a	11510 \pm 1249	8965 \pm 904	0.026 ^b
Exam	12179 \pm 1255	9349 \pm 771	9246 \pm 1333	0.047 ^b
Postexam	9437 \pm 1361	11736 \pm 1261	9479 \pm 1377	0.398
IFN- γ				
Baseline	3143 \pm 800	2334 \pm 452	1696 \pm 389	0.131
Exam	4044 \pm 732	2546 \pm 809	3640 \pm 674	0.289
Postexam	2712 \pm 515	2911 \pm 760	2564 \pm 994	0.978
IL-4				
Baseline	22.2 \pm 3.5	15.4 \pm 2.3	14.2 \pm 3.9	0.069
Exam	17.3 \pm 2.7	13.2 \pm 4.0	14.0 \pm 3.1	0.338
Postexam	12.1 \pm 2.8	14.6 \pm 3.4	19.9 \pm 5.8	0.313
IL-5				
Baseline	842 \pm 316	936 \pm 209	830 \pm 182	0.894
Exam	388 \pm 125	934 \pm 247	926 \pm 205	0.021 ^b
Postexam	293 \pm 112	926 \pm 239	1083 \pm 193	0.005 ^b

^aMean \pm SE.

^bp values reflect significance of differences between healthy and combined asthmatic subjects at the level $p < 0.05$.

TABLE 3. Th1/Th2 CYTOKINE RATIOS FROM SUPERNATANT FOLLOWING 1-DAY CULTURE OF CELLS WITH PHA/PMA COCKTAIL

	Healthy (n = 13)	Asthma I (n = 11)	Asthma II (n = 10)	Contrast (healthy vs. asthma) p
IL-2:IL-5				
Baseline	1.52 ± 0.15 ^a	1.23 ± 0.14	1.08 ± 0.08	0.029 ^b
Exam	1.73 ± 0.15	1.18 ± 0.13	1.06 ± 0.12	0.001 ^b
Postexam	1.66 ± 0.15	1.25 ± 0.15	0.95 ± 0.06	0.004 ^b
IFN-γ:IL-5				
Baseline	0.60 ± 0.28	0.43 ± 0.22	0.25 ± 0.12	0.344
Exam	1.19 ± 0.17	0.39 ± 0.21	0.67 ± 0.08	0.003 ^b
Postexam	1.06 ± 0.13	0.53 ± 0.21	0.16 ± 0.20	0.004 ^b
IL-2:IL-4				
Baseline	2.85 ± 0.08	2.91 ± 0.10	2.82 ± 0.09	0.86
Exam	2.97 ± 0.17	2.98 ± 0.14	2.83 ± 0.11	0.74
Postexam	2.82 ± 0.07	3.01 ± 0.13	2.78 ± 0.10	0.59
IFN-γ:IL-4				
Baseline	1.92 ± 0.19	2.10 ± 0.18	2.02 ± 0.09	0.49
Exam	2.43 ± 0.18	2.20 ± 0.19	2.44 ± 0.12	0.61
Postexam	2.24 ± 0.09	2.29 ± 0.12	2.05 ± 0.12	0.61

^aMean ± SE.

^bp values reflect significance of differences between healthy and combined asthmatic subjects at the level $p < 0.05$. Data are transformed by base 10 logarithm.

Blood Analysis. As expected, plasma levels of IgE indicated a significant overall group difference across the three time points: $F(2,28) = 4.24$, $p = 0.025$, respectively. The average levels of IgE were almost 10 times higher in asthmatics than in controls: $F(2,31) = 5.90$, $p = 0.007$ for Baseline, $F(2,30) = 3.63$, $p < 0.04$ during Exams, and $F(2,28) = 4.73$, $p = 0.017$ during the Postexam period, respectively (Fig. 1). Asthma II subjects also had higher mean levels of IgE than did asthma I subjects at all time points. Similarly, both the overall percentage and absolute number of eosinophils were significantly higher in asthmatic subjects than in healthy controls: $F(2,23) = 4.66$, $p = 0.02$ and $F(2,23) = 5.03$, $p = 0.015$, respectively (Fig. 1). The statistics for the absolute number of eosinophils showed that the differences were most evident during the Baseline period.

Pulmonary Function. PEFs were measured in asthmatic individuals only. The two asthmatic groups did not differ in the peak flow expiratory rates, although it should be reiterated that the asthma II group required more medication to maintain their lung function and had more males than the asthma I group. In a subset of asthmatics who measured their baseline peak flow rates in the morning and at bedtime (7 in the asthma I and 5 in the asthma II groups), the decrease in flow rates at bedtime was more prominent in asthma II subjects: -5.4 vs. -39.3 L/min.

Effect of examination stress

Cytokine Profile. Despite a number of group differences in cytokine profiles between healthy controls and asthmatic subjects, we did not find significant changes in the production of any single cytokine across the examination period. The most notable changes over time were noted in the ratios of IFN-γ:IL-5 and IFN-γ:IL-4: $F(2,44) = 5.00$, $p = 0.01$, and $F(2,44) = 6.40$, $p = 0.004$, respectively. Healthy controls demonstrated a marked increase in these cytokine ratios during the Exam pe-

riod, which was sustained above baseline values during the Postexam period, whereas this pattern was less clear in the two asthma groups (Fig. 2).

Blood Profile. The levels of IgE and eosinophil numbers and percent did not change significantly across the three time points.

Pulmonary Function. PEFs were also not affected by the examinations. The flow rates remained stable throughout the school semester.

Correlations between cytokines and blood profiles

The two Th1-type cytokines, IL-2 and IFN-γ, were significantly correlated during the Baseline and Postexam periods ($r = 0.48$, $p = 0.005$, and $r = 0.49$, $p = 0.005$, respectively), but this relationship weakened during the Exam period ($r = 0.29$, $p = 0.11$). In contrast, Th2-type cytokines, IL-4 and IL-5, were not significantly correlated at any time point. Interestingly, however, there were consistently high correlations between IL-4 and IFN-γ across the three time points: $r = 0.42$, $p < 0.02$ during Baseline, $r = 0.37$, $p = 0.03$ during the Exam period, and $r = 0.52$, $p = 0.003$ in the Postexam period, respectively.

Because of the known effects of IL-5 on eosinophil activity, we also examined the relationship between IL-5 and eosinophils. IL-5 levels were not significantly correlated with either eosinophil number or percent at any time point. Similarly, IL-4 levels were not correlated with IgE. However, IgE level was significantly correlated with eosinophil number during the Exam period, $r = 0.42$, $p < 0.02$.

DISCUSSION

The main goal of this study was to test the hypothesis that a stressful event, such as final examinations, alters cytokine production in asthmatic adolescents. Unlike the previous reports

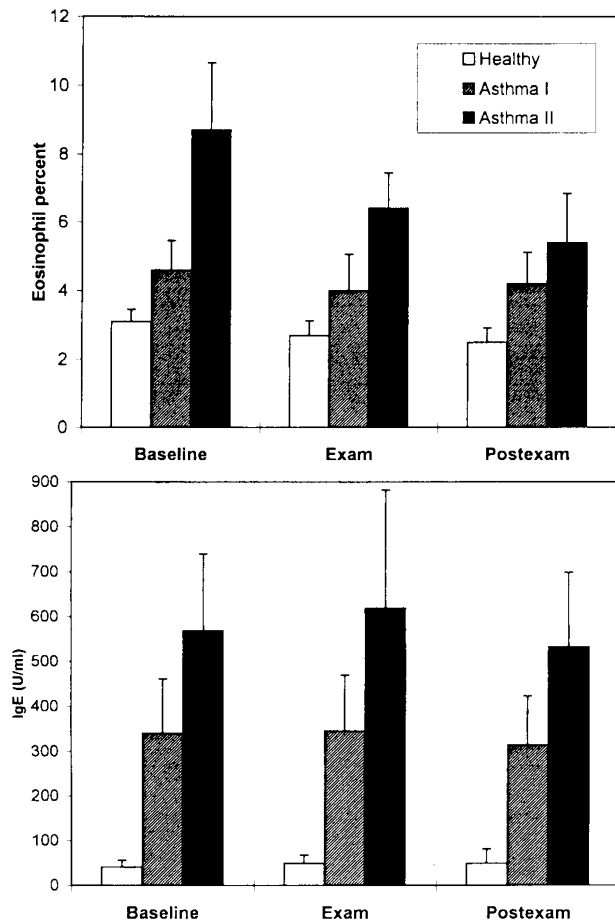


FIG. 1. Group differences in eosinophil percent (top) and IgE (bottom). Eosinophil percent differed significantly between the healthy and asthma II groups during Baseline and Exam periods. This group difference between healthy and asthma II subjects extended to IgE levels at each time point.

of a significant reduction in IFN- γ and an increase in IL-2 production by lymphocytes during the examination period in medical students,⁽¹²⁻¹⁴⁾ we did not observe any significant changes in the *in vitro* release of individual cytokines, IL-2, IFN- γ , IL-4, and IL-5, that was restricted to the examination period. Instead, the pattern of IL-5 production during both the Exam and Postexam periods was found to differ between healthy and asthmatic subjects. Healthy subjects showed a considerable decline in IL-5 production during the Exam and Postexam periods ($p = 0.055$), whereas asthmatics did not show the examination-induced decrease in IL-5. A similar pattern was found in IL-4 production. These decreases in the production of IL-4 and IL-5 appear to largely account for the sustained increases in the IFN- γ :IL-4 and the IFN- γ :IL-5 ratios in healthy subjects during the Exam and Postexam periods.

The clinical significance of this difference in cytokine pattern is not clear. Although it was not associated with a shift in pulmonary function during the Exam period, it should be reiterated that asthmatic subjects were allowed to continue their asthma medications, both steroid and nonsteroid inhalants,

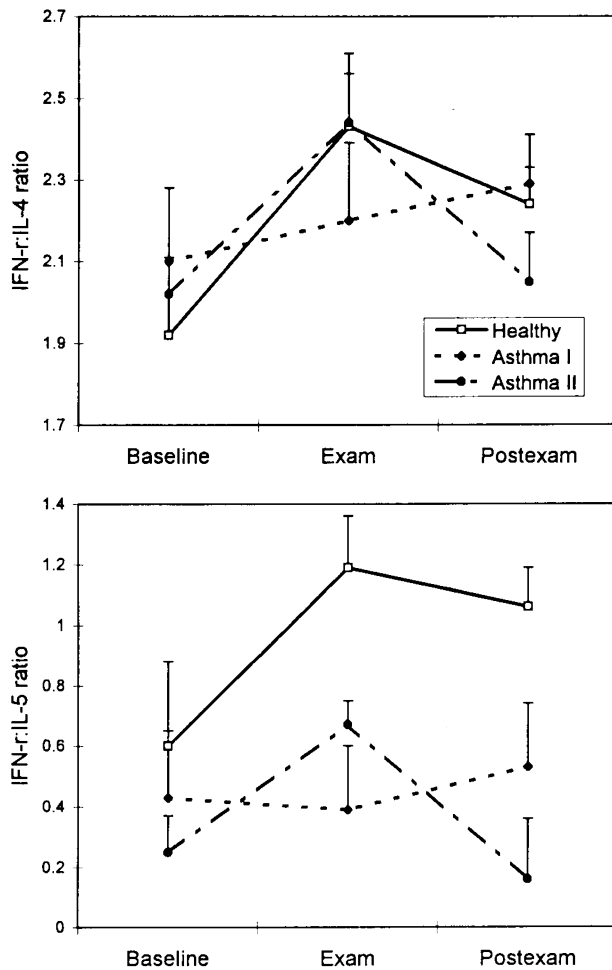


FIG. 2. Changes in the IFN- γ :IL-4 ratio (top) and IFN- γ :IL-5 ratio (bottom) over time. Significant changes were found in both Th1/Th2 ratios across the examination. For the IFN- γ :IL-5 ratio, there was a significant group \times time interaction, indicating a significantly different pattern of response over time among the three groups.

throughout the study to evaluate changes in a normative situation. Although the effects of these inhalants are mostly local, one cannot completely rule out their possible effects on peripheral blood cell function.

Overall, cytokine profiles of cells from asthmatic subjects reflected a Th2-like predominance, whereas the cell response from healthy control subjects was more indicative of a Th1-like predominance. Specifically, cells from asthmatic subjects produced significantly more IL-5 than did the cells from healthy controls across the Exam and Postexam periods. In contrast, proliferating cells from control subjects generated significantly higher levels of IL-2 during the Baseline and Exam periods following stimulation with the PHA/PMA cocktail. These patterns were supported further when the balance between Th1 and Th2 cytokines was evaluated. Healthy control subjects showed significantly higher ratios of IL-2:IL-5 as well as IFN- γ :IL-5 than did the asthmatic students.

These findings are consistent with other studies reporting a

predominant Th2-pattern in asthmatic subjects.^(20–22) Asthmatics, particularly during an acute, symptomatic period or with severe asthma, typically present a strong bias for IL-4 and IL-5 production when cells are obtained from either bronchoalveolar lavage or PB. The overproduction of IL-4, however, may be more a feature of an atopic state than a characteristic of asthma.⁽¹⁰⁾ Although IL-4 can effectively induce the production of IgE,⁽²³⁾ the correlations between peripheral blood IgE production and IL-4 level have not been consistent.⁽²⁴⁾ In our study, IL-4 levels did not directly correlate with the plasma level of IgE, nor did they correlate with IL-5 levels in the supernatant sample.

Higher IL-5 and eosinophil values in asthmatics appear to support the purported relationship between IL-5 and eosinophil function in the pathology of asthma. Other researchers have reported that the percentage of Th cells expressing IL-5 mRNA was highly correlated with eosinophil counts in asthmatics.⁽¹¹⁾ Moreover, improvement in pulmonary function following an oral glucocorticoid therapy coincided with reduced percentages of IL-5 mRNA encoding cells, as well as decreased IL-5 secretion and eosinophil count.^(11,25) In our study, however, the IL-5 levels induced from mononuclear cells were not directly correlated in a linear manner with the blood eosinophil numbers.

Although we did not find significant correlations between pulmonary function, as measured by PEF, and cytokine levels, this result may have been influenced by the relatively stable and mild form of asthma. Asthmatic students in this study appeared to be medically well managed, and the continued use of daily medication may have mitigated against any exacerbation of symptoms during the stress of examinations. The influence of stress may have been more evident if the medication had been controlled and a more severe form of asthma had been studied.

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