

ORIGINAL ARTICLE

Different inflammatory mechanisms in lungs of severe and mild asthma: crosstalk of NF-kappa-B, TGFβ1, Bax, Bcl-2, IL-4 and IgE

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Abstract

Objectives: To examine differences in the apoptotic, inflammatory, allergic and immunological features in the lungs of adults with asthma. **Material and methods:** Thirty-six patients with mild asthma (MA), 16 with severe asthma (SA) and 20 healthy volunteers (HVs) were enrolled. Bronchoalveolar lavage fluid (BALF) was processed into cell-free fluid for enzyme-linked immunosorbent assay detecting soluble TGFβ1, IL-4 and IgE and BALF lymphocytes for immunocytochemical staining of cellular Bax, Bcl-2 and nuclear factor-Kappa-B (NFκB). **Results:** Cellular NFκB expression was higher in SA than in MA and HVs, while extracellular TGFβ1 was high in both the SA and MA groups but low in the HVs. Bcl-2/Bax ratio was higher in SA than in MA and in MA than in HV groups and correlated significantly with NFκB level. Interestingly, the levels of IgE and, to a lesser extent, IL-4 were higher in MA than in SA and both were much higher than in HVs, and were inversely correlated with NFκB level in the SA group and with TGFβ1 level in the MA group. **Conclusions:** NFκB has a central role in the perpetuation of persistent inflammation in SA and might induce apoptosis via Bcl-2. The SA group appears not associated much with allergen-based IgE and IL-4 reactions as efficiently as in MA. This was supported by the lower levels of IgE and IL-4 in SA compared to MA. TGFβ1 appears to be associated with asthma pathogenesis, especially allergen-based MA.

Key Words: Allergy, apoptosis, atopy, BALF, cell survival, cytokines, ELISA, immunocytochemistry, inflammation, lymphocytes

Introduction

Asthma is a waxing and waning disease that leads to structural changes in the airways; it is a common disorder in which chronic inflammation of the bronchial tubes causes narrowing of the airways and swelling (1). According to the United States National Heart, Lung and Blood Institute, asthma is classified into: intermittent, mild persistent, moderate persistent and severe persistent (2).

Apoptosis, a form of physiological cell death, is a highly regulated and fundamental process used throughout the biological world to eliminate unwanted cells like those involved in the immune response after specific antigen clonal expansion (3,4). Therefore, apoptosis malfunctions have been involved in the chronic status of many inflammatory diseases (5), including asthma, leading to long-lasting unwanted inflammation and adverse effects of immune cell recruitment and activity (6). Bax and Bcl-2, as pro-apoptotic and anti-apoptotic factors,

respectively, have been considered as ideal markers for assessing apoptosis, especially in immunohistochemistry or immunocytochemistry assays where they can provide evidence on cellular apoptotic status after tissue fixation and cell death (4,6).

Transforming growth factor (TGFβ1) is an intercellular signalling molecule that demonstrates a plethora of biologic effects in both *in vitro* and *in vivo* contexts (7). The potential role of TGFβ1 in asthma pathogenesis has been reviewed recently and its role in asthma is still controversial (7–9). Some recent studies have shown that TGFβ1 expression increases in asthmatics (10,11), while others have found that its expression is unregulated in asthma (12,13). TGFβ1 activity is known as pro-apoptotic and fibrogenetic, and exerts a compromise of pro-inflammatory or anti-inflammatory activities depending on the phase of the disease (14).

Another factor that plays a role in pulmonary diseases is nuclear factor-kappa-B (NFκB). This

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acts as a central pro-inflammatory factor in the induction of pro-inflammatory gene expression, leading to the synthesis of cytokines, adhesion molecules, chemokines, growth factors and enzymes that inhibit apoptosis of the immune cells and induces inflammation (15). NFkB activation is a key event in a variety of cell and animal model systems developed to elucidate the pathobiology of lung diseases (16,17). On the other hand, in asthma, interleukin-4 (IL-4), which is Th2 interleukin, is found to play a major role in inducing immunoglobulin E (IgE) synthesis and allergen-oriented plasma cells stimulation (18). It is believed that IL-4 and IgE are mainly responsible for the exacerbations of asthma after allergen exposure (19).

In this study, we investigated the interplay among TGFβ1, NFkB, IL-4, IgE and apoptosis markers, Bax, Bcl-2 and bax/Bcl-2 ratio in lungs of three groups; severe persistent asthmatics (SA), mild intermittent asthmatics (MA) and healthy volunteers (HVs). The aim of this study was to elucidate the plot of asthma pathogenesis in terms of inflammation, apoptosis and allergen response between two types of asthma, namely MA and SA. Our hypothesis is that the perpetuated inflammatory reaction in severe persistent asthma diverges greatly from that in mild intermittent asthma. This is the first study exploring the differences between severe and mild asthma in adult patients in terms of inflammatory (TGFβ1 and NFkB), apoptotic (Bcl-2 and Bax) and allergic (IL-4 and IgE) factors during exacerbation periods, i.e. differences dependent on the local lymphocytes of lung tissues rather than peripheral blood lymphocytes.

Material and methods

The population of the study

Fifty-two asthmatic patients (31 M and 21 F) were involved in this study in the period February 2007 to May 2008 in Selangor, Malaysia. Their ages ranging from 17 to 53 years, with a mean of 46.3 ± 3.4 years, they had attended the respiratory clinic and ward of three main hospitals. The selection criteria were: asthmatic patients with either mild or severe asthma; no symptoms of the common cold or of any other respiratory disease at the time of exacerbation; non-smokers; devoid of any other major type of allergy, such as rhinitis, rhinoconjunctivitis, atopic dermatitis or food allergy; and no history of, or current, major illnesses other than asthma. Thirty-six patients (22 M and 14 F) presented with MA and 16 (9 M and 7 F) with SA. The MA group ranged in age from 17 to 51 years (mean 45.8 ± 3.2 years) and the SA group from 21 to 53 years (mean 47.5 ± 3.8 years). Both the MA and SA groups were diagnosed by specialists in respiratory diseases, and diagnosis was confirmed and classified by medical history

(number of attacks per month, duration of attacks, the baseline therapy, the therapy required to control attacks), physical examination at the time of presentation and review of the results of pulmonary function tests (FEV1, FVC, PEF and FEF 75%). MA patients were those with a history of between 1 and 3 mild attacks per month (mild wheezing and coughing with or without mild shortness of breath) and attacks were controlled primarily by the use of bronchodilators. Moreover, physical examinations and pulmonary function test indices (FEV1, FVC, PEF and FEF 75%) proved the mild obstructive airway pattern of asthma. On the other hand, SA patients were: those with a history of two or more severe attacks per month (harsh wheezing and coughing with severe shortness of breath); their daily activities severely restricted by frequent attacks of asthma; attacks controlled only by regular use of high doses of inhaled corticosteroids (for example, 800–1,600 μg/day of beclomethasone dipropionate). Moreover, physical examination and pulmonary function test indices (FEV1, FVC, PEF and FEF 75%) proved the severe obstructive airway pattern of asthma.

Twenty age-matched and sex-matched HVs were involved in this study, aged 23 to 55 years (mean 47.2 ± 2.5). They had no history of smoking or any medical disease, and their chest radiograms and pulmonary function tests showed no evidence of respiratory disease. Despite the difficulty of the bronchoalveolar lavage (BAL) procedure during asthmatic exacerbations, 16 SA and 36 MA patients volunteered to perform BAL. Written consent was received from the patients and from the HV group. The procedures pursued in dealing with human subjects were carried out within the scope of the Helsinki Declaration of ethical principles of medical research, and permission was granted by the Ethics Committee of Biomedical Research of University Putra Malaysia. Patients who underwent BAL were kept under strict care for their own safety; BAL was done within the first 12h of exacerbation, i.e. whenever the signs and symptoms of exacerbation were conducive as determined by the specialist.

Bronchoalveolar lavage

BALF was withdrawn from the SA, MA and HV groups by a specialist physician. For asthmatic patients, BALF was withdrawn after 8–12h from the beginning of the exacerbation. BAL was performed after local anaesthesia using commercial sterile 0.9% saline solution for intravenous use via a channel of a fiberoptic bronchoscope. BAL was performed as described by Yamaguchi et al. (20). Briefly, three 50-mL aliquots of 0.9% NaCl were instilled into a distal bronchus in the right middle lobe. Each aliquot of the infused fluid was immediately aspirated and filtered through gauze and stored. Recovered BALF

was centrifuged at 3000g for 5 min to recover cell pellets. The cell-free supernatant was stored for further analysis. Cell pellets were washed twice in sterile phosphate-buffered saline solution (PBS) (Sigma). In addition, the cell viability of BALF was determined by trypan blue exclusion. Cell differentials were counted after cytocentrifugation and staining by May-Grunwald Giemsa (200 cells on each slide). Macrophages, epithelial cells, lymphocytes, eosinophils and neutrophils were counted and the results were given in percentages and in number of cells/mL.

BALF lymphocyte isolation

BALF lymphocytes were isolated according to the method of Tsao et al. (21), with little modification. Briefly, cell pellets obtained from BALF were resuspended in 1.2 mL of sterile PBS and then spun in a density gradient ficoll. Interfaced cells were harvested and washed in PBS, then resuspended in RPMI-1640 medium (Sigma) containing 200 U/mL penicillin G, 200 µg/mL streptomycin and 10% human AB serum (Sigma) at a concentration of 1×10^6 cells/mL. Alveolar macrophages were removed by adherence to Petri dishes after incubation for 30 min in a humidified incubator with 95% air and 5% CO₂ at 37°C. The non-adherent cells were harvested by washing the dishes in warm medium three times followed by passage through nylon wool columns. Finally, cell suspension was counted and re-adjusted to a concentration of 1×10^6 cells/mL.

Antibodies

Rabbit polyclonal antibodies against cellular Bax, Bcl-2 and NFκB were used in the immunocytochemistry (ICC) slides of the isolated BALF lymphocytes; antibodies were anti-human Bax antibody (ab10813), anti-human Bcl-2 (ab18210) and anti-human NFκB p65 (ab31481) from Abcam plc, UK. On the other hand, mouse monoclonal antibodies were used against human IL-4, human IgE and human TGFβ1 in the enzyme-linked immunosorbent assay (ELISA); for the BALF cell-free supernatants, antibodies were anti-human TGFβ1 (2C5), anti-human IL-4 (NYPHIL4) and anti-human IgE (4H10) from Abcam. Biotinylated goat secondary antibodies (ab6785) against mouse immunoglobulins were used in ELISA and antibodies (ab6702) against rabbit immunoglobulins in ICC from Abcam.

Immunocytochemistry of BALF lymphocytes

The ICC procedure was conducted in accordance with the instructions of the manufacturer of primary and secondary antibodies (Abcam) with modifications; 200 µL of 1×10^6 cells/mL BALF lymphocyte

suspension was aliquoted into wells of the cytospin (these performed (350g for 6 min)) on silane-coated slides, which were then examined under the microscope to ensure that the cells had annealed properly, had normal morphology and lay flat on the slide. The slides were dried in a desiccation chamber overnight and then fixed in 2% paraformaldehyde (BDH) for 20 min at room temperature and washed three times in PBS. The slides were incubated in 95°C preheated antigen retrieval buffer, 100 mM Tris (Sigma), 5% (w/v) urea (Merck), pH 9.5 for 10 min in a water bath. After washing in PBS ($\times 3$), cells were permeabilized for 15 min at room temperature in blocking buffer, 3% bovine serum albumin (BSA) (Merck) in PBS, plus 0.1% Triton X-100 (Sigma) followed by blocking of non-specific binding in blocking buffer for 1 h at room temperature.

One hundred microlitres of the polyclonal rabbit primary antibodies against human Bax, Bcl-2 and NFκB was diluted 1:50 in blocking buffer at a final concentration of 0.005 mg/mL and applied onto ICC slides incubated overnight in a humid chamber at 4°C. Next day, the slides were rinsed gently in PBS-Tween (PBS 0.05% v/v Tween20 from Merck) and placed in a fresh PBS-Tween bath for 1 min; 100 µL of the biotinylated goat anti-rabbit secondary antibodies diluted 1:400 in PBS at a final concentration of 0.00125 mg/mL was added and incubated in a humid chamber for 1 h at 37°C. Two to three drops of streptavidin-horseradish peroxidase reagent (Dako) were then applied, and the slides were placed in a humid chamber and incubated for 30 min at 37°C. The prepared DAB-substrate chromogen solution (Dako) was applied onto sections. The slides were incubated in the dark for 20 min at room temperature. Mayer's haematoxylin stain was used as a counterstain, and the slides were dehydrated and mounted with DPX mounting fluid. The immunostained cells at moderate to intense dark brown colour were considered positive, while others were considered negative. The percentage of the positively stained cells was calculated by counting the dark brown coloured cells out of 200 total cells. Positive control, negative control and HV-negative expression slides were included in every run of ICC (Figure 1). Positive controls were the slides that showed repeatedly consistent positivity of a target marker; negative controls were produced by adding blocking buffer instead of polyclonal rabbit primary antibodies against human Bax, Bcl-2 or NFκB. HV-negative expression slides were those of the HV group that showed repeatedly consistent negative staining for a particular marker. Positive and negative controls served for testing reliability of the ICC assay, while HV-negative expression slides were used to assess the endogenous staining background of ICC assay, which was found minimal and negligible for Bax, Bcl-2 and NFκB.

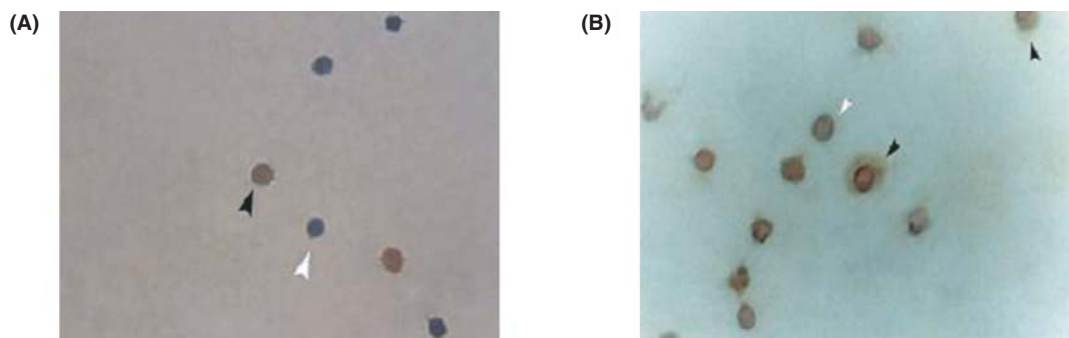


Figure 1. The immunocytochemical staining of lymphocytes with brown colour DAB-chromogen. Black arrow: Positive cells with overall; DAB Dark brown staining; white arrow: Negative cells with overall blue haematoxylin counterstaining. A. Positively immunostained cells with anti-Bcl-2 in HV. B. Numerous positively immunostained cells with Bcl-2 in SA patients. The photographs were taken at magnification power of 100 $\times$.

Enzyme-linked immunosorbent assay of cell-free BALF supernatants

Non-sandwich ELISA was modified and standardized from the routine procedure of Schuur & Weeman (22). Sterile 96 microtitre plates were coated with 50 μ L cell-free BALF supernatant 1:5 in carbonate-bicarbonate coating buffer (1.59g/L carbonate and 2.93g/L bicarbonate from Merck). BALF supernatant-coated plates were incubated overnight at 4°C. Next day the remaining fluid was aspirated and wells were washed twice in washing buffer (PBS, 1% BSA w/v from Merck, 0.05% v/v Tween 20 from Merck). Blocking buffer (PBS, 1% BSA w/v) was then added for 1h. After the washing step, 50 μ L of mouse monoclonal antibodies diluted 1:50 in the blocking buffer at a final concentration of 0.01mg/mL was added to microtitre plates and incubated for 2 h at 37°C. After the washing step, 50 μ L of the biotinylated secondary goat anti-mouse antibodies diluted 1:200 at a final concentration of 0.002mg/mL was added and the plates incubated for 1h at 37°C. After washing, 50 μ L of streptavidin-horseradish peroxidase reagent (Dako) was added and incubated for 30 min at 37°C. Finally, after washing, 50 μ L of OPD-H₂O₂ chromogen solution (Sigma) was added for 15 min. ELISA readings were immediately measured in terms of optical density (OD) using a microtitre reader A-1764A (Beckman) at a wavelength of 492nm. A standard curve was generated using serial dilutions of known amounts of IL-4, IgE and TGF β 1. The detection limits for IL-4, IgE and TGF β 1 were 0.01, 0.015 and 0.02ng/mL, respectively.

Statistical analysis

Statistics were analysed using SPSS software v. 10 and MS Excel 2000. After confirming the normal distribution pattern of both ICC and ELISA values, mean values $\pm$ SEM and parametric multivariate *t*-tests were used to evaluate the significance of difference among mean percentages of positive

expression of BALF lymphocytes NF κ B, Bax, BCL-2 and Bcl-2/Bax ratio as well as the mean concentrations of ELISA readings of cell-free BALF IgE, IL-4 and TGF β 1. Pearson's correlation coefficient was used to correlate the level of expression of the studied factors with each other in the patients of the MA and SA groups.

Results

Differential cell count of BALF

There was no significant difference in the total number and epithelial cells of BALF among the HV, MA and SA groups ($p > 0.05$). The mean percentage of macrophages was no different between SA and MA ($p > 0.05$). The mean percentage of lymphocytes and neutrophils was highest in SA followed by MA and least in HVs ($p < 0.05$). The mean percentage of eosinophils was markedly higher in the MA group than in the HV and SA groups ($p < 0.05$) and higher in SA than in HV ($p < 0.05$) (Table I).

Immunostaining of Bax, Bcl-2 and NF κ B in BALF lymphocytes

The immunocytochemical results of BALF lymphocytes showed that the mean percentage of positive cells of Bcl-2 in the SA group (38.3 ± 4.2) was higher than in the MA group (26.3 ± 2.1), which in turn was higher than in HV (21.3 ± 1.4) ($p < 0.05$) (Figure 2). On the contrary, the mean percentage of positive cells of Bax in the SA group (17.7 ± 2.2) was lower than in the MA group (23.7 ± 1.3), which in turn was lower than the highest percentage of expressed positive cells in HV (29.2 ± 1.6) ($p < 0.05$) (Figure 2). For NF κ B, the mean percentage of the positive cells in the SA group (42.5 ± 6.8) was higher than in the MA group (5.4 ± 2.9) ($p < 0.01$), while there was no significant difference between the mean percentage of NF κ B in the MA and HV groups (1.5 ± 1.4) ($p > 0.05$) (Figure 2). These results highlight how Bax decreases and Bcl-2 and NF κ B

Table I. The mean cell count (total and differential) and the mean percentage of BALF cells in HV, MA and SA patients.

Parameter	HV 10 ³ cells/mL (%)	MA 10 ³ cells/mL (%)	SA 10 ³ cells/mL (%)
Total cell count	102.3±5.4	110.1±7.8	112.7±6.4
Macrophages	78.5±4.7 (76.7±6.1)	71.8±6.2 (65.2±4.5)	67.3±7.9 (59.7±4.7)
Lymphocytes	4.6±0.4 (4.5±0.3)	5.9±0.8 (5.35±0.5)	12.7±1.6 (11.2±1.2)
Neutrophils	5.7±0.7 (5.5±0.5)	10.8±1.3 (9.8±0.8)	15.1±2.6 (13.4±1.3)
Eosinophils	0.3±0.07 (0.29±0.09)	6.3±0.4 (5.7±0.3)	3.1±0.52 (2.7±0.4)
Epithelial cells	13.2±4.3 (12.9±3.1)	15.3±3.8 (13.9±3)	14.5±4.1 (12.8±2.1)

increase with asthma severity in a significant manner. This indicated an essential role for these proteins in the different pathophysiology and inflammatory pathways that take place in different asthma types.

In addition, on calculating the Bcl-2/Bax ratio for each group of the study, it was found to be higher in the SA group (2.16 ± 0.24) than in the MA group (1.02 ± 0.14) ($p < 0.001$). For the HV group, the Bcl-2/Bax ratio was < 1 (0.72 ± 0.1), and lower than in both the SA ($p < 0.0001$) and MA groups ($p < 0.05$) (Figure 2). This suggests progressively diminished apoptosis and increased survival of the infiltrating lymphocytes in lungs of asthmatic patients with the SA mode of the disease.

Cell-free BALF soluble TGF β 1, IL-4 and IgE

ELISA readings were expressed in terms of concentration ng/mL for soluble extracellular IL-4, IgE and TGF β 1 proteins in the cell-free supernatants of BALF. It was found that the mean concentration of IL-4 in both the MA group (0.71 ± 0.063 ng/mL)

and the SA group (0.62 ± 0.075 ng/mL) was higher than in the HV group (0.021 ± 0.009 ng/mL) ($p < 0.01$), but that the difference between SA and MA was insignificant ($p > 0.05$) (Figure 3). For IgE, it was also maximally expressed in the MA group. IgE concentration in both the MA group (1.37 ± 0.11 ng/mL) and the SA group (0.91 ± 0.084 ng/mL) was higher than in the HV group (0.037 ± 0.012 ng/mL) ($p < 0.01$), but, unlike IL-4, it was significantly higher in the MA group than in the SA group ($p < 0.05$) (Figure 3). Mean concentration of TGF β 1 in BALF was markedly high and in close figures in both the SA group (0.52 ± 0.069 ng/mL) and the MA group (0.48 ± 0.087 ng/mL), which were both significantly higher than the low level of TGF β 1 in the HV group (0.073 ± 0.01 ng/mL) ($p < 0.001$) (Figure 3). Accordingly, the ELISA results of cell-free BALF outlined a tendency toward increasing levels of TGF β 1, IgE and IL-4 in the tissues of asthmatic lungs rather than in normal lungs. Surprisingly, the total level of BALF IgE showed a significant predilection for the MA group more so

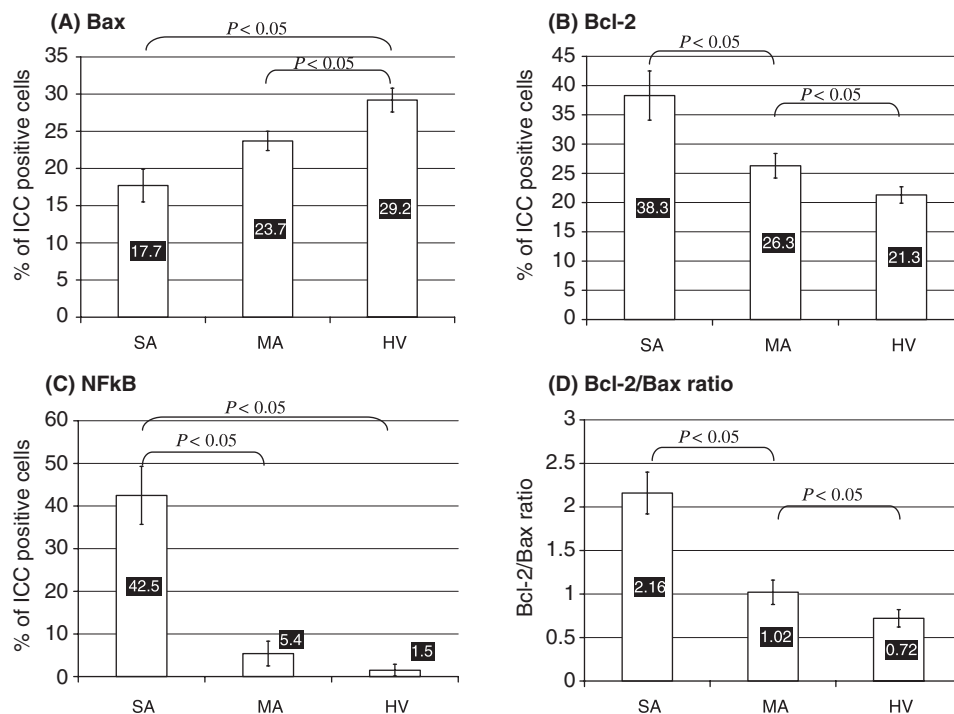


Figure 2. Mean percentage of the ICC positively immunostained cells for Bax, Bcl-2 and NFkB, as well as the mean ratio of Bcl-2/Bax in BLAF lymphocytes isolated from the SA, MA and HV groups.

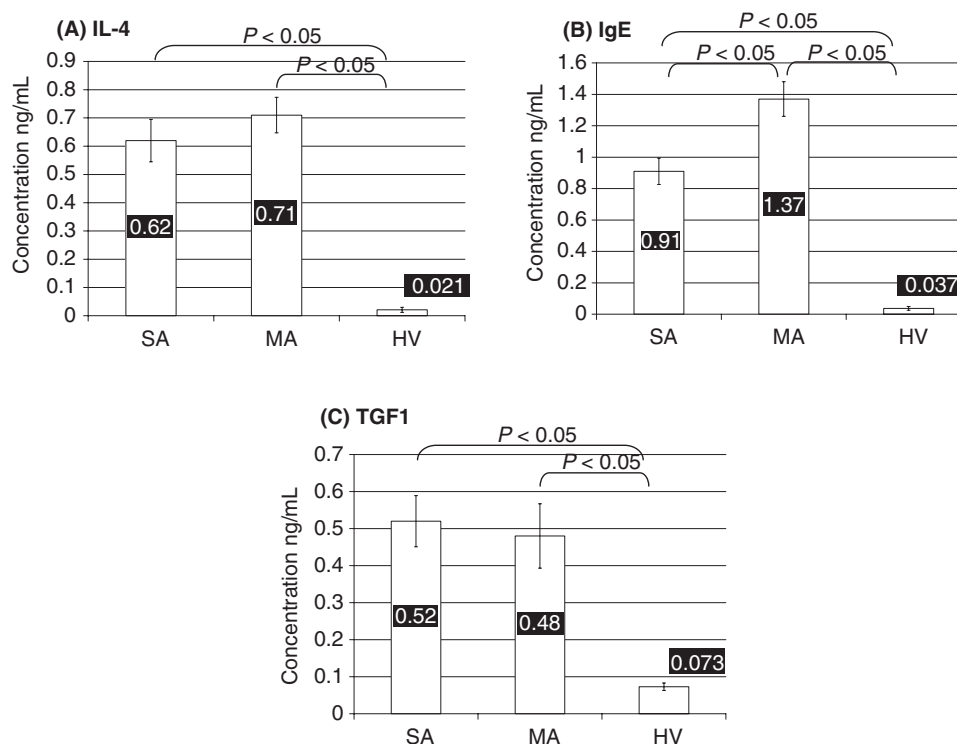


Figure 3. Mean concentrations (ng/mL) of the cell-free BALF TGFβ1, IL-4 and IgE measured by ELISA in SA, MA and HV groups.

than for the SA group, which hints at the differences in the allergen-oriented inflammatory reactions that might take place in the lungs of mild and severe asthmatic patients.

The correlation coefficient of the studied targets

The correlation coefficient was calculated among the expression level of the different proteins studied whether cellularly inside BALF lymphocytes or extracellularly as soluble factors in BALF. The correlation network was that NFκB correlated directly with Bcl-2/Bax ratio in both the SA and MA groups. Bax correlated inversely with Bcl-2 in both the MA and SA groups. IL-4 and IgE were directly correlated with each other in the MA and SA groups, but

inversely correlated with TGFβ1 in MA and NFκB in SA. TGFβ1 was directly correlated with NFκB in both the SA and MA groups (Table II).

Discussion

Asthma is unlikely to be a single disease. It is more likely to be a series of complex, overlapping individual diseases or phenotypes each defined by its unique interaction between genetic and environmental factors (23). Our study runs parallel with the conclusions of Borish & Culp (23) that the network of allergic and inflammatory factors with a role in asthma inflammation varies greatly with the disease category. The current study reflects newly clarified interactions of predisposing/propagating factors of

Table II. Correlation coefficient of the expression level of the studied factors in the SA and MA groups.

Factors of correlation	Correlation coefficient (<i>r</i>)		Correlation coefficient (<i>r</i>)	
	in SA group	<i>p</i> -value	in MA group	<i>p</i> -value
NFκB vs. Bcl-2/Bax	<i>r</i> = 0.68	<0.01	<i>r</i> = 0.59	<0.01
Bax vs. Bcl-2	<i>r</i> = -0.81	<0.01	<i>r</i> = -0.73	<0.01
TGFβ1 vs. NFκB	<i>r</i> = 0.48	<0.05	<i>r</i> = 0.52	<0.05
TGFβ1 vs. IL-4	<i>r</i> = 0.12	>0.05	<i>r</i> = -0.41	<0.05
TGFβ1 vs. IgE	<i>r</i> = 0.15	>0.05	<i>r</i> = -0.38	<0.05
IL-4 vs. IgE	<i>r</i> = 0.73	<0.01	<i>r</i> = 0.85	<0.01
NFκB vs. IL-4	<i>r</i> = -0.29	<0.05	<i>r</i> = -0.17	>0.05
NFκB vs. IgE	<i>r</i> = -0.27	<0.05	<i>r</i> = -0.13	>0.05
TGFβ1 vs. Bcl-2/Bax	<i>r</i> = 0.16	>0.05	<i>r</i> = 0.2	>0.05
IL-4 vs. Bcl-2/Bax	<i>r</i> = -0.11	>0.05	<i>r</i> = -0.16	>0.05
IgE vs. Bcl-2/Bax	<i>r</i> = -0.14	>0.05	<i>r</i> = -0.1	>0.05

asthmatic inflammation that all provide evidence of the different pathophysiology of asthmatic inflammation between severe and mild adult asthma.

Despite the difficulty, in this study BALF and BALF lymphocytes were taken from mild and severe asthmatic volunteers after 8–12 h of asthmatic exacerbations. This timing is crucial when investigating how asthmatic lungs react during exacerbations in different types of asthma, and also in minimizing the time needed by oral/inhaled asthma therapy to interfere with the findings in this type of study. The immediate anti-inflammatory action of corticosteroids is mainly caused by inhibiting the mammalian cells to respond to the inflammatory stimuli rather than reducing the inflammatory factors themselves, which need longer than 24 h (24). Moreover, the therapeutic regimens used in this study for treating exacerbations of SA and MA patients were, as far as possible, similar, using inhaled corticosteroids, in order to minimize the interfering effect of therapy. Collectively, in the light of these conditions, it is believed that the dramatic differences found between the SA and MA groups were scientifically and statistically significant and not related to the effect of therapy.

BALF lymphocytes of asthmatic lungs were targeted in this study because they are pivotal in airway inflammation in asthmatics and their infiltration into bronchial wall; interaction with inhaler antigen leads to bronchial epithelial cell damage and airways inflammation (25). BALF was used in this study because the levels of antibodies and cytokines in BALF are more sensitive and more comparable to the systemic levels than in induced sputum (26). Since both count and origin of the viable cells are a bit different in BALF from that in induced sputum (27), and since BALF is far more commonly used than induced sputum, BALF was chosen in this study so that findings were comparable with previous reports.

It was found that NFkB has a central and progressive role in perpetuating the inflammatory reactions in asthma and that its level of expression increases with the more severe types of asthma. NFkB level of expression in BALF lymphocytes was much higher in SA than in MA patients and, surprisingly, there was no significant difference in NFkB level between the HV and MA groups, even though BALF lymphocytes were taken during exacerbation periods of MA. This striking difference in NFkB expression indicates that inflammation in MA, unlike in SA, is reliant on different pathways, pathways in which NFkB does not have an important role. The exclusive high level of NFkB expression in SA explains why Bcl-2/Bax ratio doubled in SA in comparison with MA; many recent studies have shown that NFkB stimulates the expression and activity of Bcl-2, which itself acts as a potent anti-apoptotic molecule and inhibits the

pro-apoptotic Bax molecules (28–30). The results of this study concur with those of other recent studies revealing that the Bcl-2 or Bcl-2/Bax ratio is increased in asthmatic lungs compared with normal lungs (31,32), but these studies did not correlate this with different types of adult asthma. As more confirmation is added to the central role of NFkB on the apoptosis inhibition of BALF lymphocytes in asthma, NFkB has been shown to have a positive correlation coefficient with Bcl2/Bax ratio in both MA and SA patients. In addition, Bcl-2 was inversely correlated with Bax. These findings parallel the differential cell count of BALF. The higher the level of NFkB in SA leads to a higher Bcl-2/Bax ratio, which leads collectively to higher inflammatory reactions, higher number and longer survival rate of the lung inflammatory cells, namely neutrophils, lymphocytes and eosinophils. Although the percentage of eosinophils in MA and SA was higher than in HV, it was, interestingly, higher in MA than in SA. This might be explained by the lower concentrations of IgE and, to a lesser extent, IL-4 in the SA group when compared with that in the MA group, indicating that eosinophils are mainly associated with IL-4 and IgE rather than NFkB.

In this study, the level of extracellular TGFβ1 in cell-free BALF was significantly higher in both the MA and SA groups than in the HV group; its concentrations in MA and SA were very close. This finding shows that TGFβ1 is significant in the inflammatory reaction of asthmatic lungs whether the disease is still mild or persistently severe. In one study (1) it is stated that expression of TGFβ1 is altered in asthma and the current weight of evidence suggests that TGFβ1 is upregulated in human and animal asthmatic airways.

However, the expression and activity of TGFβ1 in asthma remains controversial. In some recent studies it has been shown that TGFβ1 expression increases in asthmatic patients (10,11), while in others its expression is unregulated in asthma (12,13). It is worth mentioning that the most contentious results come from studies measuring TGFβ1 expression by immunohistochemical means obtained by bronchial biopsies which require extensive tissue handling, such as fixation and antigen retrieval steps that might change the conformational integrity of the target TGFβ1. Moreover, there is no consistent cellular source of TGFβ1 in the tissues of lung. TGFβ1 is found to be secreted by airway epithelium (33,34), fibroblasts (35), endothelial cells (36) and vascular smooth muscle cells (37). Therefore, in this study, the extracellular TGFβ1 level was evaluated in the cell-free BALF regardless of cellular origin to determine its level in asthma more correctly. The role of TGFβ1 in inflammation is one of the big dilemmas in science. Up to now, there is no clear evidence disentangling the

conflicting results of the inflammatory role of TGF β 1 in general and asthma in particular. Some studies reveal that TGF β 1 exerts a protective role against excessive inflammatory reactions in pulmonary disease (38,39), while others show its potential as a pro-inflammatory and powerful chemotactic agent for neutrophils, eosinophils, macrophages and mast cells (40,41). However, the results obtained in this study might help clarify the confusion. The absence of any marked increase in the level of TGF β 1 between MA and SA patients indicates that TGF β 1, unlike NF κ B, is not associated much with the excessive inflammatory reactions seen in SA patients. On the other hand, as long as TGF β 1 is a powerful chemotactic agent and, in this study, expressed much higher in MA patients than in HVs, the scientific evidence suggests that TGF β 1 might be important in initiating an allergen-oriented inflammation in lungs of MA. This also explains why the Bcl-2/Bax ratio is not so high in MA compared with SA, because in MA there is low expression of NF κ B, anti-apoptotic, and high level of TGF β 1, pro-apoptotic. Moreover, TGF β 1 is inversely correlated with both IL-4 and IgE levels in MA but not in SA, suggesting that TGF β 1 exerts a low grade, beneficial and, under control, inflammatory response in MA. This speculation is supported by Schmidt-Weber & Blaser (14), who state that TGF β 1 induces regulatory T cells, participates directly in the suppression of effector T cells and may help return reactivity to allergens to normal sub-symptomatic activity. This scenario is strengthened by the findings of this study showing that, despite the persistent inflammation in SA patients, the level of total IgE and BALF eosinophils count in the SA group is significantly lower than in the MA group. In addition, it was found that the expression level of NF κ B in BALF lymphocytes is inversely correlated with the level of both IgE and IL-4. And since the level of NF κ B was highest in SA lungs, the level of IgE was significantly lower in SA than in MA lungs. This is actually further evidence of the divergent mechanisms and pathways for inducing and sustaining inflammation or interacting with allergens in different types of asthma. It therefore appears that TGF β 1, IL-4 and IgE play a major role in MA, while NF κ B, Bcl-2 and to a lesser extent TGF β 1 play the major role in the SA group. Previous reports have shown that IL-1, IL-6 and IFN- γ (42,43) are found in high levels in the refractory attacks of severe asthmatics, which can be explained by the findings of the current study that IL-1, IL-6 and IFN- γ are all by-products of the transcriptional activity of NF κ B (44).

Another thing that could attract attention in the results of this study, the level of both cellular expression of NF κ B and cell-free BALF TGF β 1 was highest in SA, but it seems that this did not help TGF β 1 induce apoptosis because of the stronger anti-apoptotic effect of NF κ B. Nevertheless, since

the remodelling and fibrogenetic effect of TGF β 1 is remarkable in many severe asthmatic patients (9,13), NF κ B cannot counteract the fibrogenetic potential of TGF β 1.

Conclusions

NF κ B plays an important role in perpetuating bronchial inflammation in SA but not MA patients. In addition, NF κ B might be the main factor responsible for the high Bcl-2/Bax ratio in SA leading to excessive loss of apoptosis in BALF lymphocytes and sustained inflammation unrelated to allergen inductions, as total BALF IgE was lower in SA than in MA lungs. It was also inferred that TGF β 1 is involved in the pathophysiology of mild and severe asthma but in different ways. It appears that TGF β 1 is not responsible for the severe persistent inflammation in SA patients, as its level was not increased compared to that of MA patients. On the other hand, TGF β 1, the well-known pro-apoptotic factor, seems responsible mainly for the induction of still-controllable, allergen-related, moderate inflammation in MA patients. For a better understanding, it is recommended that this study be expanded in future to include other inflammatory agents and cytokines. Moreover, a prospective study might be envisaged comparing the dynamic changes in inflammatory and allergic agents in both SA and MA during stable and exacerbation periods. Altogether, understanding the role of active factors of inflammation in each SA and MA is mandatory to help tailor asthma therapy towards each type of asthma.

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