Intracellular expression of Bcl-2 protein in lymphocytes T from children suffering from bronchial asthma

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Abstract

Introduction: Bronchial asthma is a chronic inflammatory disease of airways, characterized by persistent T cells activation in peripheral blood and airways. A disrupted immunological balance between T helper lymphocytes could be a result of an improper apoptosis process within lymphocytes. An intracellular Bcl-2 dependent pathway is one of the pathways of programmed cell death.

Aim: The aim of the paper was to evaluate the Bcl-2 expression in T lymphocytes from children suffering from bronchial asthma.

Material and methods: Peripheral blood from 25 asthmatic children, aged 8.13 ±3.08 with positive results of skin prick tests or *in vitro* tests detecting specific IgE against allergens of pollen grass, and 16 healthy children, aged 9.38 ±3.52 was analyzed. The Bcl-2 expression in T CD2+, Th1 CCR5+ and Th2 CRTh2+ lymphocytes was measured with flow cytometry.

Results. The percentage of T lymphocytes from peripheral blood of asthmatics and healthy controls was 72.8 ±8.79% and 79.07 ±6.21%, respectively, $p < 0.05$. No difference was found between the frequency of Th1 cells from asthmatic and healthy subjects, however a significant difference in the Bcl-2 expression was observed between both groups (53.82 ±30.00% vs. 75.86 ±20.11%, $p < 0.05$). The significant difference was found between the frequency of Th2 cells from asthmatic (6.51 ±4.46%) and nonasthmatic (3.01 ±1.26%) individuals, nevertheless cells did not differ in the Bcl-2 expression.

Conclusions: The imbalance between Th1 and Th2 cell subpopulations is found in asthma. An increased frequency of Th2 cells may result from a decreased Bcl-2 expression within Th1 lymphocytes and increased susceptibility to apoptosis.

Key words: bronchial asthma, Th1 lymphocytes, Th2 lymphocytes, Bcl-2, apoptosis, flow cytometry.

Introduction

Asthma is a chronic inflammatory disease of airways. Observed disorders are driven by a specialized subset of chronically activated T memory cells sensitized against an array of allergenic antigens. Asthmatic inflammation results in disturbances in bronchial walls including epithelial cell desquamation, increased myocyte proliferation, angiogenesis and thickening of lamina propria. The process of permanent structural changes in the airways is known as remodeling. Despite advances in asthma pharmacotherapy, the morbidity of the disease is still increasing [1, 2].

Granulocytes and lymphocytes' activation in peripheral blood and airways of asthmatic patients is an inherent feature of asthma. The physiological balance between T helper lymphocytes is disturbed to Th2 advantage. Cytokines released from Th2 lymphocytes (IL-4, IL-5, IL-9 and IL-12) cause IgE overproduction by lymphocytes B as well as proliferation of eosinophils and basophils in bone marrow and

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overproduction of mucus in airways. On the other hand, IgE causes degranulation of basophils and mast cells while released mediators contribute to the development of chronic inflammation in lungs [2, 3].

A persistent, disturbed balance of T helper cells could be a result of an improper apoptosis process within lymphocytes. There are 3 different mechanisms of programmed cell death. The first one is generated by signals arising within the cell, the second – triggered by death activators' binding to receptors on the cell surface (FasL, TNF-α) and the third one may be triggered by reactive oxygen species. The *bcl-2* gene family is involved in the apoptosis process as well. The Bcl-2 protein is a suppressor of programmed cell death that homodimerizes with itself and forms heterodimers with a homologous protein Bax, a promoter of cell death. The two proteins have highly similar amino acid sequences but are functionally opposed. The ratio of anti-apoptotic versus pro-apoptotic dimers is important in determining resistance of a cell to apoptosis. The Bcl-2 protein suppresses apoptosis by preventing the activation of the caspases that carry out the process. The expression of the Bcl-2 inhibits both apoptotic and in some cases, necrotic cell death in many cell types, and in response to a wide variety of inducers. Bcl-2/Bax apoptosis pathway is an important mechanism to limit an inflammatory response [4-9].

Aim

The purpose of the present study was to assess the frequency of T cell as well as T helper 1 and T helper 2 lymphocyte subpopulations in peripheral blood of asthmatic children. Furthermore, we analyzed the expression of Bcl-2 protein in T cells (CD2, CCR5 and CRTh2 positive cells) to evaluate T cell susceptibility to apoptosis.

Material and methods

Study group

Twenty-five individuals aged 8.13 ±3.08 years, 21 boys and 4 girls, suffering from atopic asthma and allergic rhinitis, sensitized to grass pollen and/or *Dermatophagoides pteronyssinus allergens*, confirmed by skin prick tests, shortlisted for specific immunotherapy, served as a study group. The study was carried out among patients treated for bronchial asthma at the Department of Pediatric Pneumology and Allergology, Medical University of Warsaw. No one of analyzed subjects has been treated with systemic corticosteroids for 4 weeks before blood collection.

Sixteen healthy individuals, aged 9.38 ±3.52 years, 7 boys and 9 girls, served as a control group. Healthy individuals were characterized by a negative quantitative IgE test and negative history of asthma. They were chosen from children undergoing routine periodical health screening with no systemic illness or recent respiratory disease. The study protocol was approved by the Ethics Committee of the Medical University of Warsaw and written consent was taken from all parents of children. Characteristics of enrolled children are presented in Table 1.

Flow cytometry

Blood was obtained by venipuncture to tubes containing EDTA (Medlab, Poland). For flow cytometric analysis, 100 μl of anticoagulated blood was stained for 25 min at room temperature in the dark using 10 μl of the following antibodies: phycoerythrin (PE)-conjugated anti-CD2 antibody (Beckman Coulter, Poland), fluorescein (FITC)-conjugated anti-CD4 antibody (Beckman Coulter, Poland), phycoerythrin (PE)-conjugated anti-CRTh2 antibody (Beckman Coulter, Poland), and phycoerythrin (PE)-conjugated anti-CCR5 antibody (Becton Dickinson, Poland). CD2 antigen is found in T cells, CCR5 antigen is a surface marker of Th1 cells [10], whereas CRTh2 antigen in the lymphocyte population is found in Th2 cells [3]. Intracellular staining for Bcl-2 was performed using Intraprep Kit (Beckman Coulter, Poland) and fluorescein (FITC)-conjugated Bcl-2 (Becton Dickinson, Poland), as per the manufacturer's instructions. Flow cytometry data were acquired by Cytomics FC500 (Beckman Coulter, USA) (Figures 1 and 2).

Table 1. The characteristics of children from study and control groups

Parameter	Children suffering from asthma (n = 25)	Healthy children (n = 16)
Age [years]	8.13 ±3.08 (5-15)	9.38 ±3.52 (3.5-16)
Gender:		
Boys	20	7
Girls	5	9
Allergic rhinitis [%]	100	0
Allergen-specific IgE (asIgE ≥ II class) [%]:		
Grass pollen	76	0
Wormwood pollen	20	0
Birch pollen	48	0
Dermatophagoides pteronyssinus	28	0
Dermatophagoides farinae	28	0

Statistical analysis

Results are presented as arithmetic mean ± standard deviation. Statistical analysis was performed using the Mann Whitey *U* test. A *p* value of less than 0.05 was considered significant.

Results

The Bcl-2 expression in CD2 positive cells

The quantitative analysis of CD2 lymphocyte frequency demonstrated a significant difference between groups of asthmatic and healthy children, 72.8 ±8.79% and 79.07 ±6.21%, respectively, $p = 0.0276$.

The analysis of Bcl-2 positive T cell frequency did not demonstrate any significant difference. In the group of children suffering from atopic asthma, 41.28 ±23.00% of T cells showed an expression of Bcl-2 antigen, whereas in the control group, 43.89 ±13.22% of CD2 positive cells showed the Bcl-2 expression, $p > 0.05$ (Figure 3).

The Bcl-2 expression in CCR5 positive T lymphocytes

In peripheral blood of asthmatic children, Th1 cell frequency was 14.72 ±6.45% whereas it was 16.86 ±7.47% in the group of healthy children, $p > 0.05$.

As shown in Figure 4, the frequency of Th1 cells expressing Bcl-2 antigen differs significantly between analyzed groups; it is 53.82 ±30.00% in the group of asthmatics versus 75.86 ±20.11% in the control group, $p = 0.02$ (Figure 4).

The Bcl-2 expression in CRTh2 positive T lymphocytes

The frequency of Th2 cell in peripheral blood of asthmatic children was 6.51 ±4.46%, whereas the frequency

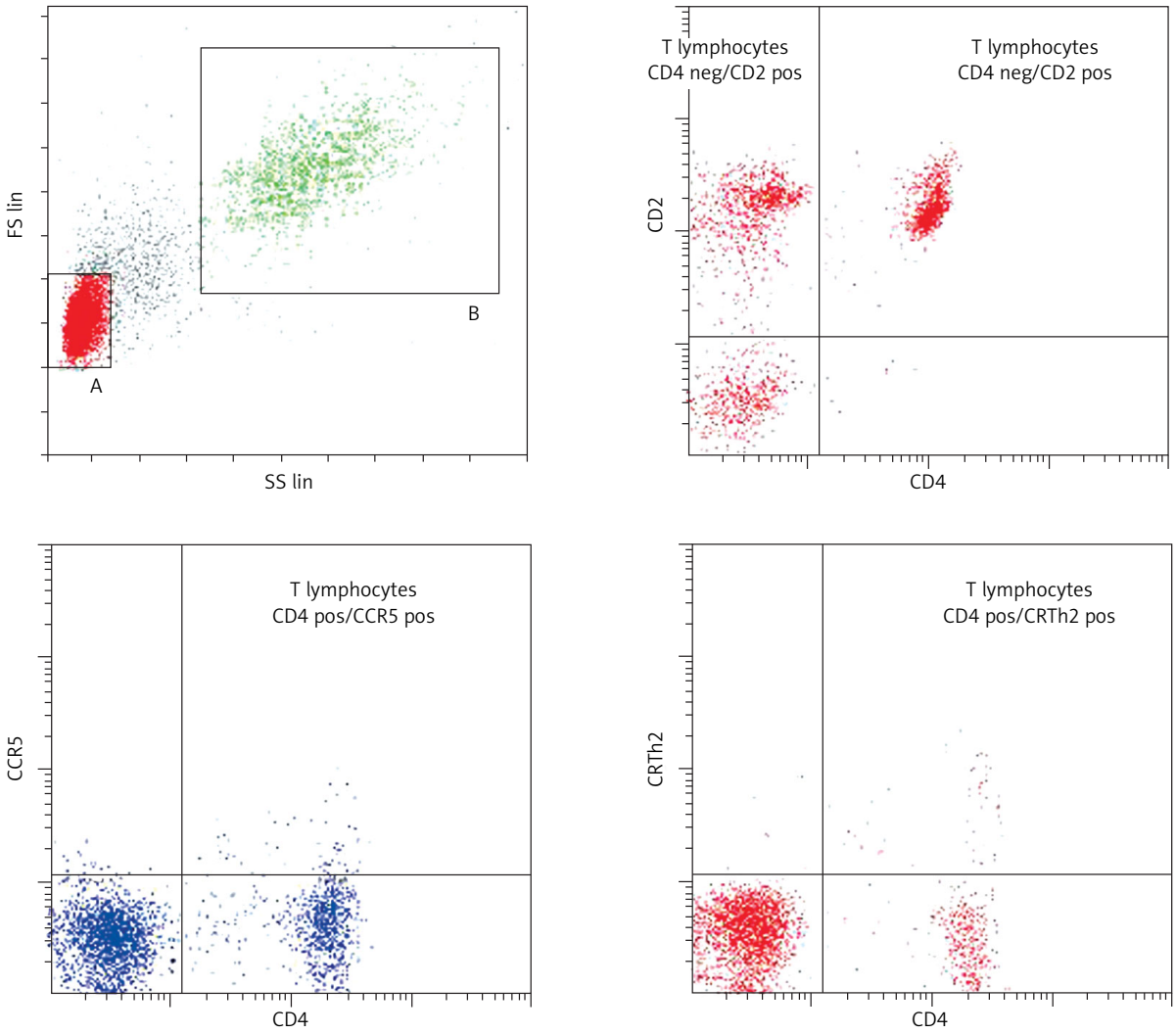

Figure 1. CD2, CRTh2 and CCR5 expression in CD4+ lymphocytes. Double positive populations are marked in the figure

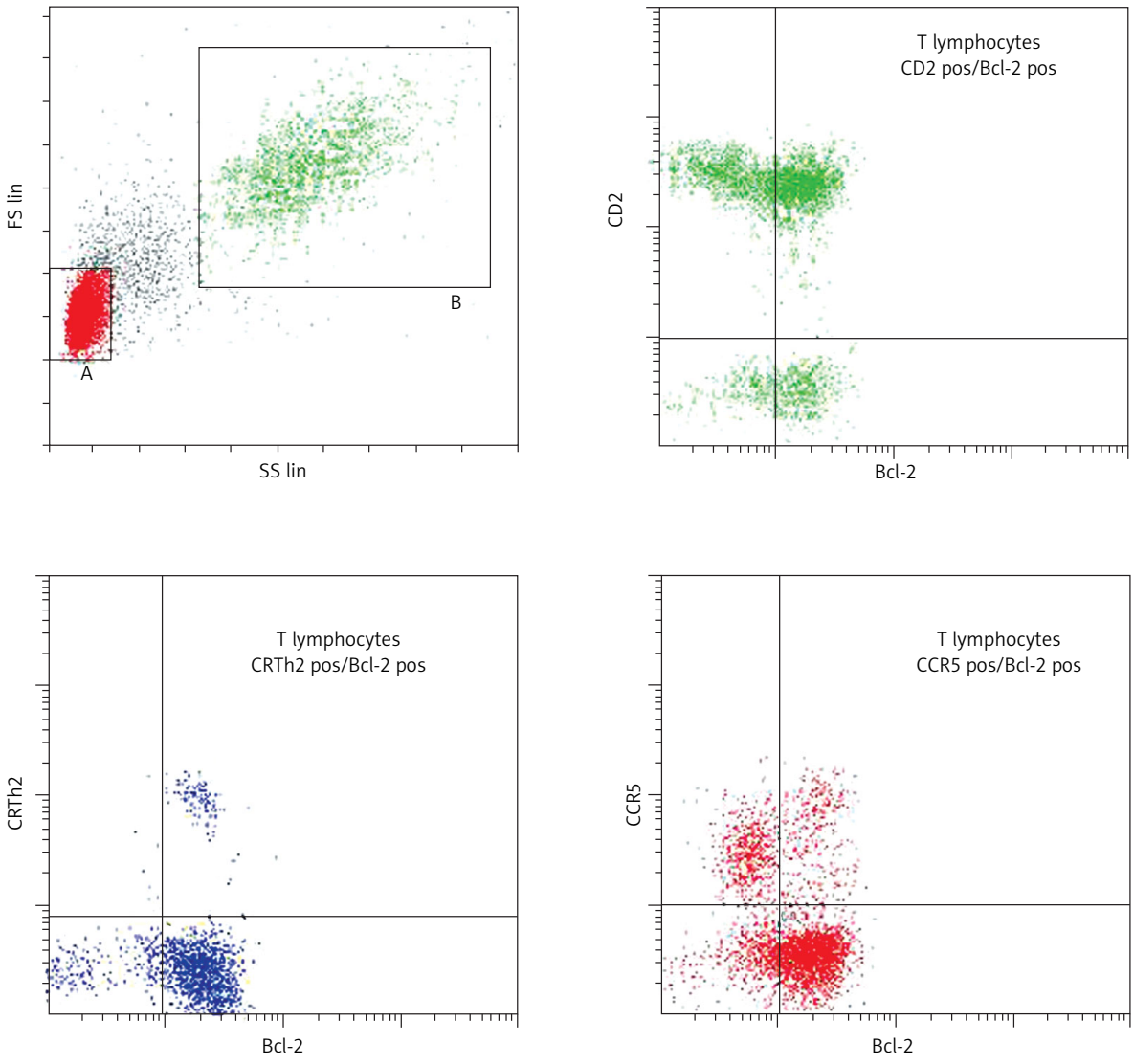

Figure 2. Bcl-2 expression in CD2+, CCR5+ and CRTh2+ lymphocytes. Double positive (T/Bcl-2+, Th1/Bcl-2+ and Th2/Bcl-2+) populations are marked in the figure

of Th2 cells in the control group was 3.01 ±1.26%; the difference is significant (p = 0.004).

The quantitative analysis of Bcl-2 positive Th2 lymphocyte frequency demonstrated a significant difference between groups of asthmatic and healthy children, 63.64 ±25.71% and 68.08 ±9.33%, respectively, p > 0.05 (Figure 5).

Th1/Th2 ratio

A significant difference in Th1/Th2 cell ratio was found between analyzed groups. In the group of asthmatics, Th1/Th2 ratio was 3.53 ±2.65, whereas in the group of healthy children the ratio was 5.51 ±2.90, p = 0.0421 (Figure 6).

Discussion

Experimental and clinical data indicate that an increased frequency of Th2 cells and an imbalance in Th1/Th2 subpopulations are major features of bronchial asthma. In healthy people, naïve T cells differentiate mainly to Th1 cells. The frequency of Th2 cells is lower, however both populations stay in a dynamic balance. It

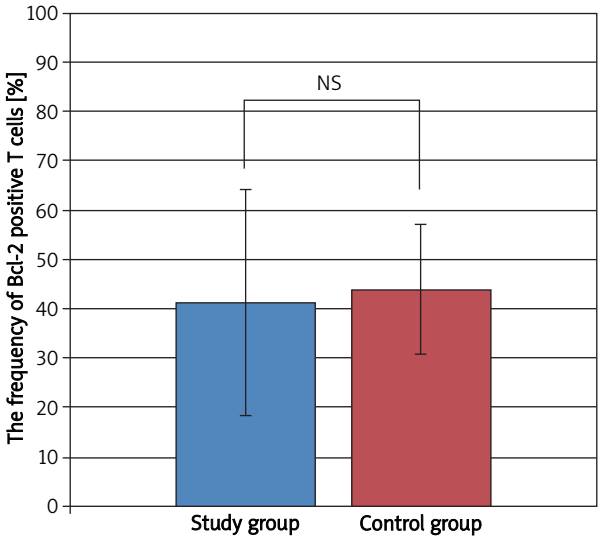

Figure 3. The analysis of frequencies of Bcl-2+ T cells in peripheral blood of asthmatic and healthy children

NS – the difference is not significant

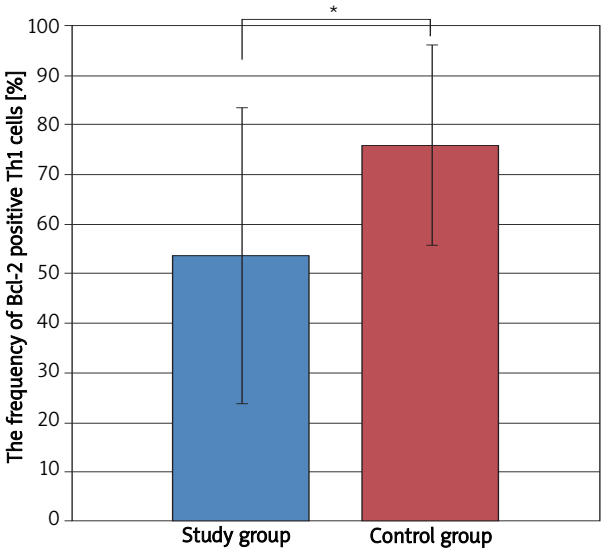

Figure 4. The analysis of frequencies of Bcl-2+ Th1 cells in asthmatic and healthy children

**p < 0.05*

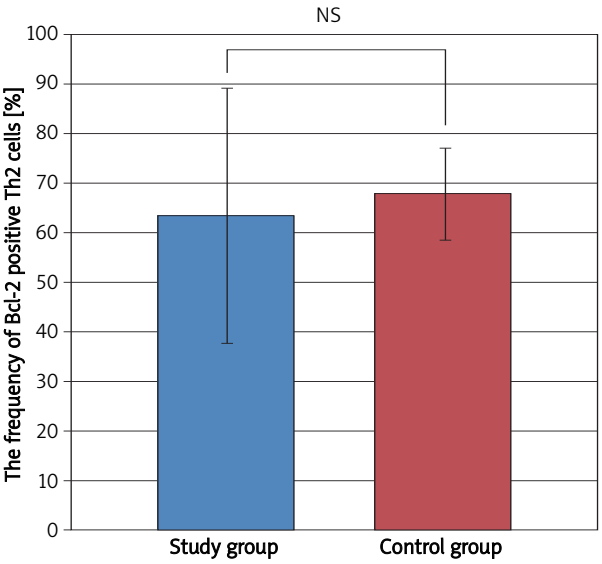

Figure 5. The analysis of frequencies of Bcl-2+ Th2 cells in asthmatic and healthy children

NS – the difference is not significant

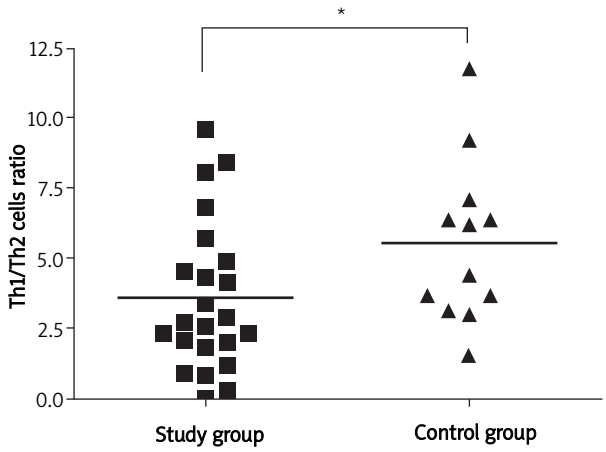

Figure 6. Th1/Th2 cell ratio in asthmatic and healthy children

**p < 0.05*

ensures harmonious development of immunological response. The disturbance of such balance is found in asthmatic patients, with Th2 cell majority [3, 7, 11, 12].

Our results confirm that the stability of Th1/Th2 ratio in the course of asthma is decreased, as compared with healthy subjects [13, 14]. In the peripheral blood of asthmatic children, an almost double increase in Th2 cell frequency was observed, in comparison with the control group. No differences in Th1 cell frequency was found. However, the relative number of Th1 cells was higher than that of Th2 cells. Other reports stay in line with our observations regarding the relative number of T helper cells in peripheral blood of asthmatics [15, 16]. It is postulated that the number of Th1 cells increases proportionately to the disease severity [16].

It is proposed that the imbalance between T helper cells in asthma results from disturbances in the apoptosis process within T cells [3-9]. Bcl-2 protein is one of the key molecules in the programmed cell death pathway. When homodimerized, it protects the cell against apoptosis, although in a complex with Bax protein – it triggers the

death process. The protective role of Bcl-2 is associated with maintaining the cell in G0 phase of the cell cycle, and preventing caspase 3 activation. Bax/Bcl-2 complex activates the cell cycle and triggers programmed cell death [5].

The assessment of the Bcl-2 expression in T lymphocytes was investigated several times. However, the results of studies are dissimilar. Abdulamir *et al*. reported that the Bcl-2 positive lymphocyte frequency was increased in peripheral blood of asthmatics, in comparison with healthy subjects. Moreover, they showed that the expression of anti-apoptotic protein increases in line with asthma severity [17]. Similarly, other authors demonstrated an increased Bcl-2 expression in lymphocytes isolated from asthmatics sputum, comparing to the healthy control group [4]. The overexpression of Bcl-2 protein is indicative for decreased susceptibility to apoptosis. It might suggest that lymphocytes from patients suffering from asthma are resistant to apoptosis triggered in the intracellular Bcl-2/Bax pathway. On the other hand, Druilhe *et al.* reported no difference in the Bcl-2 expression in T cells between asthmatic and non-atopic subjects. Noteworthy, they analyzed lymphocytes isolated from bronchial biopsies [18]. Our results confirm the observations of that study. We did not find any differences in the Bcl-2 expression in a complete T cell population from peripheral blood of asthmatic individuals suffering from controlled asthma. Analogous findings were observed when analyzing Bcl-2 concentration in lymphocyte lysates from asthmatic and healthy subjects [19]. On the other hand, Ho *et al.* reported a decreased Bcl-2 expression in T cells from peripheral blood of asthmatics, comparing to controls [20].

The dissimilarity in these outcomes of studies regarding the expression of Bcl-2 protein in lymphocytes from asthmatic subjects may result from the different material analyzed. It is beyond doubt that the T cell activation level depends on various factors, e.g. cytokines in the extracellular matrix. In this respect, the direct comparison of results of studies performed in material obtained from the bronchial biopsy, sputum, bronchoalveolar fluid, peripheral blood or lymphocyte lysates is found to be pointless.

The contact with allergen is a key factor influencing the level of expression of proteins involved in lymphocyte's apoptosis. In the present study, tests were performed beyond the pollen season, to limit exposure to allergens. Patients sensitized against house dust mite resided in the hypoallergenic environment, with limited house dust mite presence as well. Though, the expression of Bcl-2 was not affected by action of specific allergens. This information is important since it has been discovered that the Bcl-2 expression in lymphocytes increases after the allergen challenge [21].

Our data show differences in T helper cell frequencies from asthmatics, which suggests that apoptosis disturbance might be found in isolated subpopulations of T cells, not in the entire population. We demonstrated that the Bcl-2 expression in Th1 cells from asthmatic children is significantly different to that in cells from healthy children. A decreased expression of anti-apoptotic marker suggests that cells are more susceptible to programmed cell death than cells from the control group. Here we confirm that in the course of atopic asthma, the imbalance between T helper cells origin from increased apoptosis susceptibility of Th1 cells [11, 22, 23]. Other authors characterized apoptotic cells by the surface expression of phosphatidylserine, which is a marker of the advanced stage of the apoptotic process. It does not explain how programmed cell death was initiated. We pointed to the intracellular pathway involving Bcl-2 protein family. Nevertheless, it does not rule out that other apoptosis pathways are activated as well.

Our observation regarding a decreased Bcl-2 expression in Th1 cells in the course of asthma is pioneer. Although other groups observed an increased Th1 susceptibility to apoptosis, they analyzed different parameters – phosphatidylserine or Fas expression on the cell's surface, caspase 3 activation or DNA defragmentation visualized by TUNEL method [11, 23, 24].

Similarly, a decreased Bcl-2 expression in Th1 cells was proved in uremic and AIDS patients. However, these diseases are non atopic, despite the increased frequency of Th2 cells in the lymphocyte population [24, 25].

Conclusions

The disturbance in T helper cell balance and increase in Th2 cell frequency are the main features of bronchial asthma. Nevertheless, Th2 cells' domination is not associated with increased apoptosis resistance of Th2 cells, but rather increased susceptibility of Th1 cells to programmed cell death. The knowledge about the mechanism driving immunological disturbances in atopic diseases may contribute to better understanding of asthma pathogenesis and development of more effective methods of therapy.

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