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Lymphocytes apoptosis in patients with acute exacerbation of asthma

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ASTHMA is characterized by airway inflammation, which can be now assessed by the analysis of induced sputum. Ten patients with asthma were investigated during acute exacerbation for the quantification of apoptosis, for Bcl-2 and Fas expression, in induced sputum lymphocytes. They were compared to 12 patients with chronic obstructive pulmonary disease (COPD), and 10 healthy controls. Spontaneous apoptosis was determined by staining nuclei with propidium iodide, and analyzed with a FACScan. Bcl-2 was measured by Western blotting, and results were obtained by densitometric scanning, done by the gel proanalyser. The investigation of Fas was performed using the streptavidin-biotin preroxidase-complex method. Patients with asthma and patients with COPD exhibited a significant increase of cellularity, percentage of neutrophils, eosinophils and lymphocytes when compared to healthy controls. Apoptosis in induced sputum mononuclear cells was found decreased in patients with asthma compared to COPD patients and healthy controls. The quantification of apoptosis was measured after exposure to anti-cytokine antibodies. Anti-TNF-α antibody blocked the apoptosis in both patients groups and healthy controls, suggesting that TNF-α acted as an inducer of apoptosis. Anti-IL-10 blocked apoptosis completely exclusively in patients with asthma. Bcl-2 expression was found to be increased in induced sputum mononuclear cells from patients with asthma, compared to healthy controls and patients with COPD. Expression of Fas could be detected in patients with asthma, at a lower level than COPD patients and healthy controls. Distinct mechanisms of apoptosis were found in patients with asthma and patients with COPD, characterized by different levels of Bcl-2 and Fas expression. Induction of apoptosis should be a beneficial process in allergic inflammation traduced in induced sputum mononuclear cells. The apoptosis process is assumed by two different mechanisms in asthma and COPD. Our findings indicated that in asthmatic patients, activated lymphocytes accumulate in the bronchi; because of their prolonged survival that maintains inflammation.

Key words: Asthma, Chronic Obstructive Pulmonary Disease, Apoptosis, Lymphocytes, Bcl-2, Fas

Key Indexing Term: *Apoptosis and related proteins in Asthma and COPD*

Introduction

Infiltration of the airways mucosa with activated inflammatory cells appears to be a major factor in the pathogenesis of asthma and other forms of chronic obstructive airway disorders, such as chronic obstructive pulmonary disease (COPD). The cells and molecular markers of this inflammation have been studied by bronchoalveolar lavage (BAL) and bronchial biopsies but the use of these techniques has been limited in certain patients by their invasiveness. In order to overcome the difficulties associated with bronchoalveolar lavage and the sampling of bronchial biopsies via the bronchoscope, analysis of induced sputum has recently been suggested for repeated evaluation of airway inflammation in patients with asthma. Examination of induced sputum allows investigation the airway inflammation directly without important secondary effects. Inflammatory markers can be studied on cells and supernatants.

Inflammation plays a key role in the pathophysiology of asthma[1] and COPD. In bronchial biopsies of patients with asthma, primarily eosinophils, monocytes/macrophages and T-lymphocytes[1,2] are seen. In lobar bronchial biopsies of subjects with chronic bronchitis, an increased number of leukocytes, both in the epithelium and in the lamina propria, consisting predominantly of macrophages and activated T-cells, was found. Eosinophils are the more prominent cells in asthma, and their activation is supposed to be involved in tissue destruction. Epithelial bronchial cells are now considered essential in modulating bronchial tissues remodeling (subepithelial fibrosis, muscular hyperplasia). However, T-lymphocytes are still essential in asthma inflammation as the TH2 cytokines are characteristic of allergic pathways and particularly of asthma. Moreover, the major drugs used in asthma (steroids) are powerful immunosuppressors, reflecting importance of the immunologic pathways.

Homeostasis of cell number is achieved by balancing the proliferative and anti-proliferative states of cells. Anti-proliferative states include growth arrest, differentiation, senescence (cellular ageing), and apoptosis. Apoptosis, a dynamic process involved in the control of the 'tissue load' of immune effector cells at inflamed sites, tends to limit inflammatory tissue injury and to promote resolution rather than progression of inflammation.[3] The inflammatory process could be traduced by certain proto-oncogenes, which regulate the programmed cell death: apoptosis. Fas and the members of the Bcl-2 gene family have emerged as key regulators of the apoptotic process.[4] In bronchial biopsies, the number of apoptotic eosinophils and macrophages was found to be lower in subjects with asthma than those with COPD and inversely correlated with the clinical severity of asthma,[5] reflecting longer survival of these cells.

As lymphocytes are involved in the initial events of recruitment and activation of inflammatory cells, we supposed that they have to disappear to allow inflammation resolution. The aim of our study was to investigate bronchial lymphocytes apoptosis in acute asthma exacerbation. The cells were recovered on induced sputum. In this work, we will discuss how the use of sputum, and the analyses of apoptosis, promise to provide new insights into understanding inflammatory airway diseases.

Materials and methods

Patients

Induced sputum samples were collected from 10 successive patients with asthma, and 12 patients with COPD (Table 1). All were inpatients, admitted for an acute wheezing exacerbation of dyspnea, usually by the emergency services. The sample was obtained the day after their admission. A precise history of the patient was subsequently obtained, and after recovery functional respiratory tests and eventually skin tests were realized. The following patients were excluded: concomitant infectious pneumonia, tuberculosis,

Table 1. Clinical manifestations and treatment. A: asthma; B: COPD; PY: pack year; GS: glucosteroids; y: yes; n: negative results; asthma severity: 2: mild, 3: moderate, 4: severe.

Patients	Age	Smoking PY	Atopy	Reversibility		Skin tests	Dyspnea history (years)		Asthma severity	Productive cough	Previous treatment			Previous hospitalisations (number)
				Beta 2 test	GS test		Acute dyspnea	Persistant dyspnea			B2 agonists	Theophylline	GS	
A1	47	30	y	p		p	8	0	3	y	y	y	y	2
A2	22	1	y	p		p	3	0	2	n	n	n	y	1
A3	65	40	n	p		n	8	3	4	n	y	y	y	4
A4	47	0	y	p		p	11	7	4	n	n	n	n	1
A5	47	0	y	p			5	0	2	n	y	y	n	1
A6	80	0	n	n	n	n	10	0,1	3	y	n	y	y	2
A7	31	0	y	n	p	p	26	1	4	n	y	y	n	2
A8	30	15	y	p		p	12	0	2	n	y	y	n	2
A9	28	0	y	n	p	p	6	0	3	y	y	y	y	3
A10	46	10	y	p		p	29	3	4	y	n	y	y	2
B1	88	49	n	n	n	p	20	9		n	y	y	n	1
B2	71	124	n	n	n	n	0,2	0,2		y	n	n	n	1
B3	59	20	n	n	n		20	5		y	y	y	y	3
B4	68	100	n	n	n		0	8		y	n	n	n	1
B5	59	75	n	n	n	p	10	1		y	y	y	n	3
B6	65	20	n	n	n	p	10	2		y	y	y	y	1
B7	61	30	y	n	n		13	13		y	n	y	n	1
B8	52	60	n	n	n	n	15	3		n	n	n	y	1
B9	82	20	n	n	n	n	3	0		n	n	n	n	1
B10	64	100	n	n	n	n	40	1		y	y	y	n	3
B11	63	69	n	p	p	n	15	0,1		y	n	n	n	1
B12	80	80	n	p		n	1	0		y	n	n	n	1

interstial lung diseases, bronchiectasis, lung cancer, and associated acute pathologies: cardiac, renal, liver, or neurological diseases.

Ten induced sputum from healthy subjects (mean age 28.7 yrs; range 22–36), who had normal pulmonary radiographs and showed no clinical signs of respiratory diseases, acted as controls. Informed consent was obtained from all the patients. The study was approved by the local Ethics Committee.

Sputum induction

After the inhalation of salbutamol (2 × 200 µg), subjects were asked to inhale sterile, pyrogen-free, hypertonic saline in increasing concentrations for a duration of 10 min. The hypertonic saline was nebulized via an ultrasonic nebulizer. Subjects were encouraged to cough throughout the procedure. Most patients were able to expectorate an adequate sample (7 ml and more) within the first 10 min.

Sputum processing

In order to reduce salivary contamination, plugs were selected and transferred into an Eppendorf tube. Freshly prepared 10% solution of dithiothreitol (1 ml) (DTT) was added. The tube was vortex mixed and the sputum was incubated for 5 min at room temperature, filtered through 52 µm nylon gauze to remove debris and mucus, and subsequently centrifuged at 450 ×g for 10 min. The cell pellet was resuspended in phosphate-buffered saline (PBS) in a volume equal to the sputum plus DTT solution volume. Total cell counting was carried out in a haemocytometer and the cell concentration was adjusted to 1.0×10^6 cells/ml. Cytospins were prepared by adding 75 µl cell suspension into Shandon II cytocentrifuge cups (Shandon Southern Instruments) and spun for 8 min at 500 rpm. Two slides were stained with Wright-Giemsa for an overall differential cell count of leukocytes, bronchial epithelial cells and squamous cells. Slides were coded and counted blind by two investigators. None of the cytospins contained >5% squamous epithelial cells. For cell differentiation, 400 nucleated cells per slide were counted (Table 2) and expressed as the percentage of intact round nucleated cells, excluding the squamous epithelial cells.

Quantification of apoptosis

Lymphocytes were isolated by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and washed twice with PBS at 4+°C, as we have recently reported.[5] Cell pellet expressed more than 80% CD3-positive cells as determined by anti-CD3 monoclonal antibody (Becton Dickinson). These T-cells were double labeled with anti-CD3 and anti-CD25 monoclonal antibodies in original (Becton Dickinson).

Table 2. Subjects' cell characteristics.

Cells	Asthma	BPCO	Healthy controls
Number of cells × 10^6	2.5 (1,3–2,7)	1.9 (0,9–2,1)	1.4 (0.8–1.8)
Viability %	84 (45.5–94.5)	86 (56.5–92.7)	83.6 (75.6–92.0)
% macrophages	52.56 (40.3–62.9)	49.63 (40.4–59.3)	42.8 (22.7–65.0)
% neutrophils	32.85 (25.2–50.5)	35.49 (24.8–46.5)	28.0 (19.8–41.4)
% eosinophils	5.7 (2.7–9.5)	4.79 (1.6–8.1)	1.7 (0.6–2.9)
% lymphocytes	7.45 (4.2–12)	8.73 (3.0–14.5)	3.9 (0.5–4.3)
% epithelial cells	1.1 (0.2–5.6)	0.9 (0.4–2.3)	1.6 (1,4–6,7)

Apoptosis was quantified on lymphocytes by staining nuclei with propidium iodide (PI) and analysing fluorescence with a FACScan (Becton Dickinson. Mountain View, CA). Briefly following culture for 18 h, lymphocytes were collected after centrifugation with RPMI 1640 at 150 g for 10 min. The pellet was washed twice in complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml benzyl-penicillin, 100 µg/ml streptomycin and containing 10% fetal calf serum). The pellet collected from induced sputum was gently suspended in 0.5 ml of hypotonic fluochrome solution (0.1% sodium citrate with 0.1% Triton X-100) containing 20 mg per µl PI for 2O min. RNAse A at a concentration of l0 mg/ml was added and cells were further incubated for 10 min at 4°C. The suspension was analyzed by flow cytometry to determine PI fluorescence of individual nuclei. Apoptotic nuclei appeared as a broad hypodiploïd DNA peak that was easily discriminated from the narrow peak of nuclei with normal (diploid) DNA content. Student's *t*-test was used for statistical analysis of data and $p<0.01$ was taken as significant.

Detection of Fas by immunochemistry

For measuring the expression of the Fas-antigen, induced sputum cytospins were fixed with ice-cold acetone for 10 min. Immunocytochemical investigation of Fas was performed using the streptavidin-biotin preroxidase-complex method. Cell preparations were pre-incubated with hydrogen peroxide (0.3%, 15 min), unlabeled streptavidin (dilution 1:50, 15 min) and non-immune normal sheep serum. After each step slides were washed three times in PBS. The Fas-antibody antibody (Immunotech, France) was used in a dilution

as indicated by the manufacturer and applied overnight at 4°C. After three washes in PBS the cells were incubated for 45 min with biotinylated sheep anti-mouse IgG (Amersham; diluted 1:50 with 5% normal serum). After washing three times with PBS, the streptavidin-biotinylated peroxidase complex (Amersham, 1:100, 30 min) was added and subsequently washed with PBS (three times). Peroxidase activity was made visible with 3-amino 9-ethycarbazole (20 min), which gives a red-brown reacting product. Counterstaining was performed with haematoxylin and sections were mounted with glycerol gelatine. Negative controls were obtained, firstly, omitting the primary antibodies and secondly, by an irrelevant antibody. For Fas-antigen, the percentage of stained cells was estimated for each slide and the samples were classified as cells with low Fas expression if less than 1.5% (mean value) stained cells were present and as high Fas-expression samples if 1.5%or more of the cells were stained.

Expression of Bcl-2 by western blotting

The expression of Bcl-2 in induced sputum lymphocytes was determined by Western blotting. Equal amounts of protein were loaded on SDS-PAGE and blotted onto nitrocellulose paper. The Bcl-2 protein was detected by rabbit anti-human Bcl-2 antibody (Santa Cruz Biotechnology, CA) and anti-rabbit peroxidase conjugate as secondary antibody with diaminobenzidine as substrate. Densitometric scanning of the blot was done by the gel proanalyser from Media Cybernetics.

Statistical analysis

Values are presented as mean value (SEM). Differences between groups are analyzed using the Mann–Whitney U-test. Probability values of $p<0.05$ were considered significant. Reproducibility of sputum cell counts was examined by repeated-measures. Values >0.70 indicate high reliability.

Results

Patients

According to clinical history, functional (spirometry, and reversibility with beta2 agonists or steroids) and skin tests results, patients were classified as asthmatic patients or COPD. Chronic asthma severity was classified according to GINA recommendations.[6] Three patients were difficult to diagnose definitely as asthma or COPD (A1, B11 and B12) but we decided not to exclude them, the results obtained on their samples reflecting a real association of the 2 diagnosis.

Differential cell counts

Differential cell count in induced sputum was reported in Table 2. Patients with COPD and patients with asthma exhibited a higher total cell number, an increased percentage of macrophages, eosinophils and neutrophils when compared to healthy control induced sputum ($p<0.01$). Patients with asthma and patients with COPD exhibited significant increased levels of lymphocytes when compared to healthy controls ($p<0.01$).

Quantification of apoptosis

Induced sputum lymphocytes from patients with asthma, patients with COPD and healthy controls, showed spontaneous apoptosis (mean ± s.e.m.) after 18 h of culture of lymphocytes in the absence of mitogens (Figure 1). These lymphocytes were CD3- and CD25-positive. The percentage of apoptosis was significantly decreased in patients with asthma (11.37% ± 5.31 %; $p<0.01$) compared with COPD patients (14.82% ± 4.46%), and healthy controls (26.09% ± 12.34%).

Pathogenic concepts of allergic diseases include a central role for differential cytokine production, characterized as Th0/Th2 profile. Then we tested if certain anti-cytokines antibodies could block the apoptosis in patients with asthma, with COPD and in healthy controls.

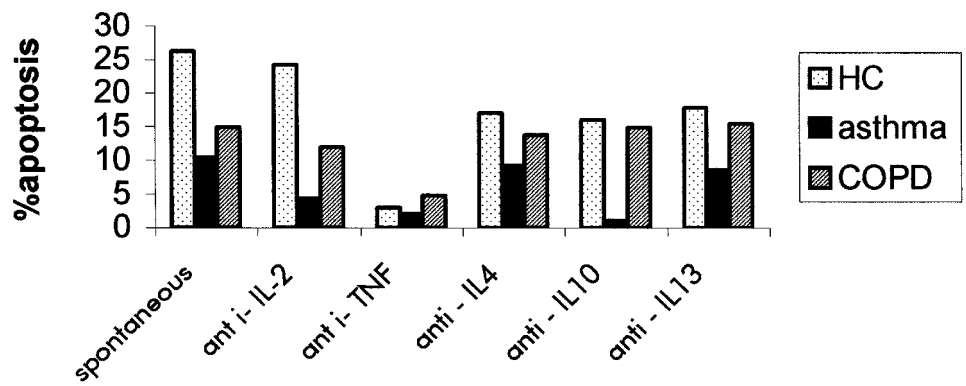

FIG. 1. Percentage of apoptosis in patients with asthma (n = 10), in patients with COPD (n = 12), and in healthy controls (HC) (n = 10).

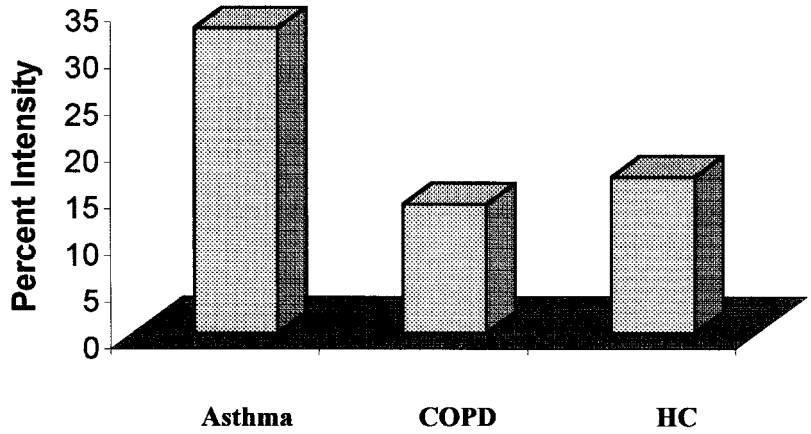

FIG. 2. Expression of Bcl-2 in induced sputum mononuclear cells from 10 patients with asthma, 12 patients with COPD, and 10 healthy controls (HC). Densitometric scanning of the Western blot quantifies the expression of Bcl-2.

Induced sputum lymphocytes were cultured in the presence of various anti-cytokine antibodies, anti-IL-2, anti-IL-4, anti-IL-10, anti-IL-13, and anti-TNF-α (Fig. 1). Anti-IL-2 antibodies inhibited the apoptosis both in patients with asthma and patients with COPD by 45% and 7% respectively. Anti-IL-10 blocked apoptosis exclusively in patients with asthma by 70%. Anti-TNF-α antibody inhibited the apoptosis in both patients groups and healthy controls, suggesting that TNF-α acted as an inducer of apoptosis.

Expression of Bcl-2 in induced sputum mononuclear cells

The analysis of Bcl-2, which is the apoptosis inhibiting protein, was estimated by Western blotting. The results were highly reproducible. The concordance of the repeated experiments was for Bcl-2 measurement of 87%.

The expression of Bcl-2 was found to be increased in induced sputum lymphocytes for patients with asthma (32.7% ± 7.4%) compared to healthy controls (16.8% ± 6.2%) and patients with COPD (13.9% ± 2.8%) ($p<0.01$) (Fig. 2). The Bcl-2 level was not significantly different in patients with COPD, and healthy controls. The Bcl-2 levels were also detected by two-color flow cytometry analysis (anti-CD3 and anti-Bcl-2 monoclonal antibodies) and similar results were obtained. Cells with light scatter characteristics of lymphocytes were gated and analyzed using LYSYS II software from FACScan flow cytometer (Becton Dickinson) (data not shown).[7]

Expression of fas in induced sputum mononuclear cells

We analyzed 10 samples of induced sputum from patients with asthma, from patients with COPD ($n=12$) and from healthy controls ($n=10$) for Fas expression. The percentage of stained cells was estimated for each slide. The results were represented individually for each patient (Fig. 3). A significantly

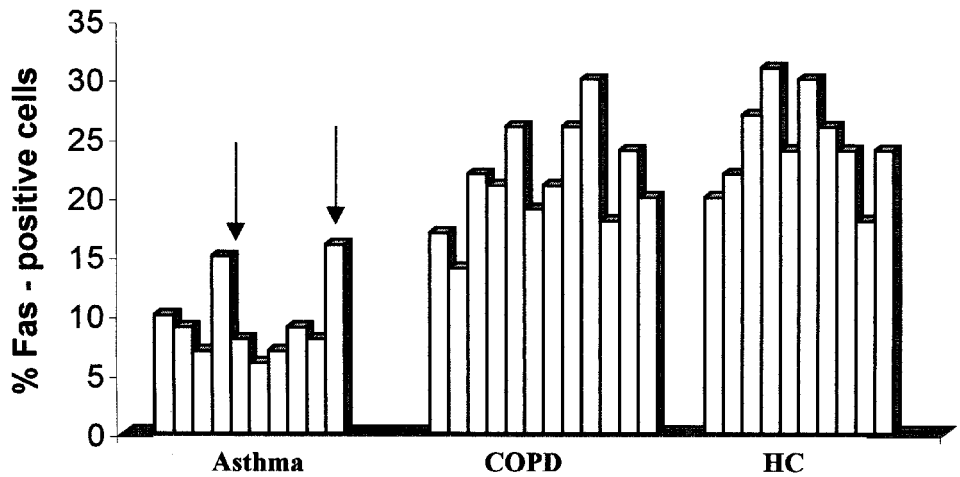

FIG. 3. Expression of Fas in induced sputum mononuclear cells from 10 patients with asthma, 12 patients with COPD, and 10 healthy controls (HC). Individual values were expressed in percentage (number of Fas-positive cells/number of mononuclear cells × 100). Asthmatic patients number 4 and 10 [→], have subnormal values of Fas-positive cells. According to Table 2, they have low values of eosinophils.

low expression of Fas could be detected in patients with asthma, when compared to COPD patients and to healthy controls ($p<0.01$).

Discussion

Total cell count was elevated in patients with asthma and patients with COPD, compared to healthy controls. Sputum of patients with asthma and COPD expressed high percentages of eosinophils and lymphocytes. The lymphocytes were in the majority CD3-positive (T-lymphocytes), and were activated ($CD25^+$). The leukocytes count and phenotype of the lymphocytes showed that sputum traduced an inflammatory state in patients with asthma and COPD. The results obtained from analyzing induced sputum in asthma and COPD patients reflect the inflammatory status of the bronchi.

The percentage of apoptosis was significantly decreased in patients with asthma compared with COPD patients and healthy controls. Anti-IL-2 antibodies inhibited the apoptosis both in patients with asthma and patients with COPD. Anti-IL-10 blocked apoptosis exclusively in patients with asthma. Anti-TNF-α antibody inhibited the apoptosis in both patients groups and healthy controls, suggesting that TNF-α acted as an inducer of apoptosis.

The expression of Bcl-2 was found to be increased in induced sputum lymphocytes from patients with asthma compared to healthy controls and patients with COPD.

A significantly low expression of Fas could be detected in patients with asthma, when compared to COPD patients and to healthy controls.

No correlation was found with asthma severity, or previous treatment. This can be linked to our protocol: all the samples were collected during an acute exacerbation requiring an admission. We can suppose that the severity of the acute process was similar within the patients and did not depend on the severity of the chronic disease. It would only depend on the pathology: asthma or COPD.

In an attempt to clarify if lymphocytes survival is modified in asthma, we investigated induced sputum samples. The percentage of apoptosis was significantly decreased in patients with asthma compared with COPD patients and healthy controls. The decreased apoptosis observed in patients may be associated with persistence of mononuclear cells in the bronchial mucosa and lumen, during the acute exacerbations. The low expression of apoptosis is more important in patients with asthma, when compared to COPD patients. This finding suggests a particular involvement of lymphocytes in acute asthma. Induction of apoptosis allowing the removal of the cells may be essential to resolve allergic inflammation, and the use of corticosteroids and theopylline in combination may be appropriate to induce apoptosis in eosinophils and lymphocytes.[8]

Interestingly, Vignola et al.[5] found significantly more apoptotic cells in the biopsy specimens of patients with chronic bronchitis than in tissues from asthmatic patients. The authors reported also[5] that there is an imbalance between the expression of Bcl-2 and p53, which appears to be more important in asthma than in chronic bronchitis. They concluded that airway inflammation in asthma is associated with an enhanced survival of different cell types caused by reduced apoptosis. Our findings corroborate their findings, but we focused on lymphocytes. The percentage of intensity of the proto-oncogene Bcl-2 was increased more in patients with asthma when compared to COPD and to the healthy control group. The Fas proto-oncogene which is involved in the induction of apoptosis was significantly decreased in patients with asthma, when compared to COPD patients and to healthy controls. If the Fas is involved in the induction of apoptosis at the same level as p53, our reports were in accordance with those of Vignola et al.[5] The Bcl-2 was found to be increased in the tissues from asthmatic subjects compared with those of the control subjects, and the expression correlated with the severity of asthma.[5] Although Bcl-2 can be associated with resident cells (mucous gland and smooth muscle), both protein and message were specifically found in T-cells of the inflammatory infiltrate in subjects with asthma and chronic bronchitis.[9] Upregulated Fas ligand may trigger the apoptotic death of Fas-bearing cells. This study reported the down expression of Fas in lymphocytes in sputum of asthmatic patients. Functional Fas receptor expression on eosinophils varies among individuals with hypereosinophilia, and the survival cytokine.[10,11] We have found that certain specific anti-cytokines antibodies blocked apoptosis, in particular anti-TNF-α and anti-IL10 in patients with asthma. Anti-TNF-α inhibited apoptosis in two patients groups and healthy controls. The blocking of apoptosis with anti-TNF-α antibody indicated that TNF-α is involved in inducing cell death.[12,13] This correlates well with the findings that serum levels of TNF-α increase with the severity of disease.[14]

Incubation of lymphocytes from asthmatic patients with anti-IL-10 antibody decreased apoptosis more significantly than in patients with COPD or healthy controls. This shows that IL-10 had a specific role in asthma. We have recently reported that IL-10 is able to increase in vitro expression of Bcl-2 in peripheral blood lymphocytes and in inflammatory lymphocytes from broncho-aveolar lavage and cerebrospinal fluid.[7] It has been reported that continuous culture of T-cells in the presence of IL-10 will inhibit T-cell apoptosis, because of, at least in part, the upregulation of Bcl-2.[15] In patients with asthma, anti-IL-10 antibody abolished apoptosis in asthma. This may have a particular

significance. Anti-IL-10 could have a different effect on sputum T-lymphocytes or the action of anti-IL-10 was different, as has been observed *in vivo*, or *in vitro* long-term culture (more than 48 hours). IL-10, a product of T helper 2 lymphocytes, has been shown to be an important regulator of peptide and alloantigen-induced T-cell proliferation and IL-2 production.[16] Human IL-10 exerts a dose-dependent inhibitory effect on human stimulated lymphocytes *in vitro*.[16]

Interleukin-2 is a major T-cell growth factor, and anti-IL-2 antibody inhibit apoptosis.[17] In the present report anti-IL-2 antibody inhibited apoptosis more in asthma and COPD than in healthy controls. This finding could be explained by the fact that T-lymphocytes in sputum of patients played an inflammatory role and were probably producing high levels of inflammatory mediators. The anti-cytokines data further substantiate that IL-2 and IL-10 are critical factors during inflammatory process in the lung.

Apoptosis signals are further modulated by inhibitors or inducers of apoptosis including Bcl-2, p53, Fas-ligand. Further understanding of the interaction of these molecules in asthma and COPD may lead to more specific therapies for immunosuppression tailored to the genetic or environmentally induced, activation-induced apoptosis defect in patients with asthma.

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