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Increased apoptosis of peripheral blood mononuclear cells in patients with perennial allergic asthma/rhinitis: relation to serum markers of apoptosis.

Janina Grzegorczyk Ph.D.,[CA,1] Marek L. Kowalski M.D., Ph.D.,[2] Anna Pilat Ph.D.,[2]* and Jolanta Iwaszkiewicz Ph.D.[2]

[1]Janina Grzegorczyk Ph.D.; Department of Clinical Immunology and Allergy, Faculty of Medicine, Medical University of Lodz; 251 Pomorska St., 92-213 Lodz, Poland; [2]Department of Clinical Immunology and Allergy, Medical University of Lodz, Poland *Central Clinical Hospital, Lodz, Poland

[CA] Corresponding Author
Tel: + 48 42 678 15 36
Fax: + 48 42 678 22 92
E-mail: ngrzegor@csk.am.lodz.pl

***Background*: The goal of our study was to examine spontaneous and stimulated apoptosis of peripheral blood MNC from allergic patients, sensitized to Der p I antigen as compared to cells from non-atopic subjects. Furthermore we aimed to investigate which populations of mononuclear cells (lymphocytes, monocytes) undergo the apoptosis and to determine relations between apoptosis and serum levels of sFas/APO-1, ICE/caspase-1 or TNF-α.**

***Methods*: The study included 17 patients with perennial, allergic asthma and/or allergic rhinitis [6 male and 11 female; mean age 29,5 years; (range 15–49)].**

Apoptosis was assessed by fluorescence technique and confirmed by flow-cytometric method and DNA ladder. Serum levels of sFas, ICE/caspase-1 or TNF-α were determined by immunoassays (ELISA).

***Results*: Apoptotic index of unfractionated mononuclear cells (MNC) and lymphocytes (but not monocytes) were significantly higher in allergic patients as compared to non-allergic subjects after 48 and 72 hours of culture ($p<0.05$). Incubation of cells with ConA (10 μg/ml) resulted in a significant increase in the proportion of apoptotic cells in all populations once the apoptotic index for MNC and lymphocytes (but not monocytes) was again significantly higher in allergic as compared to non-allergic subjects after 24, 48 and 72 hour of culture.**

In allergic patients, mean serum sFas level, was significantly lower then in non-allergic group (mean value 624.8 pg/ml ± 25.67 versus 802.0 pg/ml ± 31.91; $p = 0.003$) and in both groups sFas level correlated inversely with apoptosis of MNC. The mean ICE/caspase-1 concentration was significantly higher in sera of allergic patients as compared to non-allergic group (mean value 27.71 pg/ml ± 3.79 vs. 23.54 pg/ml respectively; $p<0.01$). ICE/caspase-1 levels in allergic patients correlated with apoptotic index of mononuclear cells ($r = 0.57$; $p<0.001$).

***Conclusions*: An increased spontaneous and mitogen-induced apoptosis of MNC from peripheral blood of atopic patients as well as different serum levels of sFas and ICE/caspase-1 correlating with apoptosis, suggest different regulation of apoptotic process in peripheral blood mononuclear cells of patients with allergic asthma and/or rhinitis.**

Abbreviations: MNC-mononuclear cells; PCD- programmed cell death; TNF- tumor necrosis factor; ICE/caspase-1 – Interleukin 1β converting enzyme; ConA – Concanavalin A; PI – Propidium iodide;

Key words: Atopic asthma, apoptosis, allergic inflammation, mononuclear cells, sFas/APO-1, TNFR, caspases.

Introduction

Mononuclear cells (MNC) including monocytes and T-cells, constitute a proportion of cellular infiltrate in the asthmatic airway mucosa and play a significant role in the pathogenesis of allergic airway inflammation.[1] Both monocytes and T-cells are the source of several cytokines and chemokines with proinflammatory activity stimulating and perpetuating allergic inflammation.[2,3,4,5,6] Development of chronic inflammation involves the absence of balance between proliferation and elimination of the

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cells. Elimination of cells may occur by two different mechanisms: necrosis and apoptosis. Necrosis is a pathologic form of cell death resulting from acute cellular injury, and typified by rapid cell swelling and lysis. Shrinkage, nuclear condensation, and membrane blabbing and membrane changes that lead to phagocytosis of the affected cell characterize apoptosis (programmed cell death; PCD).[7] Recent data suggest that mechanisms involved in the regulation of the survival and apoptosis of inflammatory cells may play a central role in the persistent inflammatory process characterizing allergy and asthma. However, most studies focussed so far an apoptosis of eosinophils, al thought survival of lymphocytes may be also an employed factor regulating allergic inflammation.[8]

During activation by specific antigen, T helper lymphocytes synthesize several growth factors e.g. IL-2, which induce proliferation of specific T lymphocytes. Although IL-2 prevents apoptosis by regulation of synthesis proteins such as Bcl-2 and Bcl-x_L, during specific activation some T cells undergo apoptosis. Triggering of specific cellular receptors, such as TNF receptor superfamily, can also induce PCD.[9,10,11] The pivotal functions in apoptotic pathways play **C**ytosolic **A**spartate-**S**pecific **P**roteases, called Caspases. They are present in all cells as latent enzymes and are recruited to receptor-associated cytosolic complex, which is formed by initiation of receptor oligomerization (e.g., TNF-receptors, FAS, TRAIL). During the activation caspases can initiate a cascade of intracellular events leading to apoptosis. ICE/caspase-1 (Interleukin-1β?converting enzyme) was the first member of the caspase family to be identified as a novel type of cysteine protease responsible for the conversion of precursor interleukin-1 to its mature form in monocytes. The mature form of IL-1β claved at Asp-116-Ala-117 is a key mediator of inflammation.[12,13] Programmed cell death play a significant role in immunological processes such as transplantation and graft-versus-host reaction but in allergic diseases the role of apoptosis in unclear yet.

The goal of our study was to examine spontaneous and stimulated apoptosis of peripheral blood MNC from allergic patients, sensitized to Der p I antigen as compared to apoptosis of cells from non-atopic subjects. Furthermore we aimed to investigate which populations of mononuclear cells (lymphocytes, monocytes) undergo the apoptosis and to determine relations between apoptosis and serums levels of sFas/APO-1, ICE/caspase-1 or TNF-α.

Patients and methods

Patients

The study included 17 patients with perennial, mild bronchial asthma and/or allergic rhinitis [6 male and 11 female; mean age 29.5 years (range 15–49)]. All patients had positive skin prick tests with Der p I antigen and some of them were also sensitized to other seasonal or perennial allergens. All asthmatic patients were on inhaled steroids (not exceeding 200 μg of budesonide), but none of them had taken oral corticosteroids. Methyloxantins or shorts acting antihistamines were stopped at least 72 hours before the blood samples were obtained. (Table 1) The control group comprised 16 subjects without any symptoms or history concerning the respiratory tract and with negative skin prick tests to a battery of inhalant allergen.

Table 1. Characteristics of allergic patients.

NR	Initials	Sex	Age in years	Clinical diagnosis
1	R. A.	F	28	Rhinitis
2	P. A	F	47	Asthma + Rhinitis
3	K. G	M	21	Asthma + Rhinitis
4	W. Z	M	40	Asthma + Rhinitis
5	B. R	M	19	Asthma + Rhinitis
6	K. P	F	18	Rhinitis
7	N. R	F	22	Asthma + Rhinitis
8	B. A	F	30	Asthma + Rhinitis
9	J. I	F	20	Asthma + Rhinitis
10	P. M	F	38	Asthma + Rhinitis
11	D. A	F	49	Asthma + Rhinitis
12	F. A	F	22	Asthma + Rhinitis
13	C. S	M	19	Asthma + Rhinitis
14	S. A	F	29	Asthma + Rhinitis
15	K. W	F	47	Asthma + Rhinitis
16	W. W	M	39	Rhinitis
17	K. B	M	15	Asthma + Rhinitis

Cells isolation

Mononuclear cells were isolated with Boyüm's method.[14] In brief: 20 ml heparinized venous blood were mixed with PBS in 1:3 proportion, then carefully stratified on Histopaque 1.07 g/cm^3 (Sigma, Germany) and centrifuged at 400 $\times$g for 200 min. The ring which was formed on the borderline of the phases was carefully collected and rinsed in phosphate buffer (pH 7.4 without Ca^{++} and Mg^{++}). Finally, suspended in the medium supplemented with 0.3% albumin, Ca^{++} and Mg^{++}, and 0.036% glucose so that the number of cells equaled 2×10^6/ml. One aliquot of MNC suspension was cultured. The second was used to assay cell surface markers. The composition of unfractionated PBMC was tested by immunofluorescence method with monoclonal antibodies: anti – CD2, CD3, CD4, CD8, CD22, CD56, CD14 (DAKO, Holland). The residual portion cells from 12 out of 17 patients were further isolated into lymphocytes and monocytes by adherence on plastic dishes. Briefly: the mononuclear cells were resuspended in medium enriched with antibiotics (penicillin – 100 U/ml and

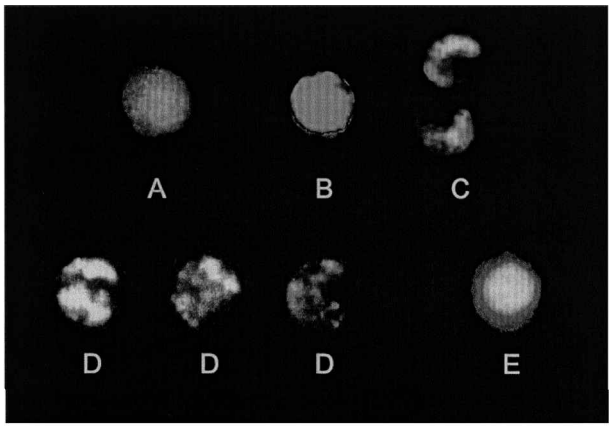

FIG. 1. Apoptotic morphological changes in mononuclear cells stained with orange acridine and ethidium bromide. A – alive lymphocyte; B – D different morphological changes in apoptotic lymphocytes; E – necrotic lymphocyte. (Fluorescence microscope; 200x).

streptomycin – 10 μg/ml) at final concentration of 2×10^6/ml. Five milliliter aliquots of cells were incubated in plastic dishes at 37°C in 5% CO_2 for 1 hour. Non-adherent cells were removed by vigorous washing and the adherent cells by scraping with rubber policemen. The adherent cells were typically >65% monocytes, as assessed by immunofluorescence method with monoclonal anti-CD14 antibody[15] and the remaining cells were lymphocytes.

Cell culturing and assessment of apoptosis

Unfractionated MNC (both lymphocytes and monocytes) were cultured[16] for indicated period of time with ConA (10 μg/ml) or with the medium alone. The samples were preincubated (with or without ConA) for 4 h in the incubator with 5% CO_2 and 95% humidity at 37°C. Following preincubation, cells were washed twice in PBS without Ca^{++} and Mg^{++}, suspended in the primary medium volume, and cultured for 24, 48 and 72 hours.

The MNC were removed from culture after 24, 48 and 72 hours to study apoptosis by the fluorescence technique, as described McGahon.[17] Twenty-five μl aliquots of MNC were mixed with PBS solutions containing 100 μg/ml of acridine orange base (Sigma) for assessment of nuclear morphologic characteristics and 100 μl/ml ethidium bromide (Sigma) for assessment of cellular viability. Stained MNC were transferred to a glass slide and examined by means of fluorescence microscope. Minimum of 200 total cells was counted and the number of apoptotic cells with highly condensed chromatin was recorded. (Fig. 1). The percentage of apoptotic cells (apoptotic index) was calculated as follows:

$$\%\ \text{Apoptotic cells} = \frac{\text{Total number cells with apoptotic nuclei} \times 100}{\text{Total number cells counted (viable+apoptotic +necrotic cells)}}$$

Flow-cytometric determination of apoptosis

MNC from 6 subjects (3 atopic asthmatic patients and 3 healthy persons) were examined by cytofluorymetry. Annexin V-FITC- 5 μl and PI-10 μl (PharMingen, USA) were used to quantitatively determine the percentage of cells undergoing apoptosis [18] The following controls were used to set up compensation and quadrants: unstained cells; cells stained with Annexin V-FITC (no PI); cells stained with PI (no Annexin V-FITC).

Isolation of apoptotic DNA fragments

Fragmentation of DNA was assessed according to Herman.[19] The MNC were removed from culture after 72 hours. After harvesting MNC samples were washed with PBS and pelted by centrifugation. The cell pellets were then treated for 10s with lysis buffer (1% NP –40 in 20 mM EDTA, 50 mM Tris-HCL, pH–7.5; 10 μl per 10^6 cells). After centrifugation the supernatants were brought to 1% SDS and treated for 2 h with RNAse at 56°C. Followed by digestion with proteinase K for at last 2 h at 37°C. After addition of ½ vol. 10M ammonium acetate, the DNA was precipitated with 2.5 vol. ethanol. Next, separated by electrophoresis in 1-% agarose gel containing ethidium bromide (i.e. 35V for approximately 4h). The samples were visualized by UV illumination and analyzed by gel analyzing system (Vilber Lourmat, France).

sFas/APO-1, ICE/caspase-1 and TNF-α measurment in serum

The serum in the same sample of blood, were collected and frozen at –20°C until the time of determination of sFas/APO-1 (PharMingen); ICE/caspase-1 and TNF-α (Bender MedSystems) by ELISA method.

Statistical analyses

Wilcoxon's or F tests were applied to statistically analyze the data. Spearman's test and regression were used to assess relation between level sFas/APO-1, ICE/caspase-1, TNF-α and baseline time (T_0) apoptosis.

Results

We did not observe any significant differences between MNC surface markers in allergic and non-allergic subjects (data not shown).

Table 2. Spontaneous and Con A stimulated apoptosis of MNC in allergic (n = 12) and non-allergic subjects (n = 16).

	Allergic patients			Non-allergic subjects		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
MNC Spontaneous	19.33 ± 1.48	33.58 ± 3.59*	43.33 ± 4.02*	16.81 ± 1.36	21.21 ± 21.6	26.59 ± 2.38
Con A	47.7 ± 3.96	54.3 ± 4.07	54.67 ± 4.02	35.28 ± 12.55	39.14 ± 2.9	40.0 ± 3.57
Lymphocytes Spontaneous	9.66 ± 0.99	20.25 ± 2.2*	28.33 ± 3.93*	9.00 ± 0.76	14.7 ±1.07	19.87 ± 1.79
Con A	51.66 ± 2.79*	55.83± 2.21*	49.91 ± 5.22*	31.42 ± 1.37	33.42 ± 2.06	31.85 ± 2.89
Monocytes spontaneous	28.33 ± 2.7	31.5 ± 2.4	36.58 ± 3.78	27.03 ± 2.88	33.96 ± 2.84	35.9 ± 3.53
Con A	54.58 ± 2.21	59.5 ± 2.96	57.33 ± 3.48	39.57 ± 2.79	40.0 ± 3.09	40.85 ± 3.85

* Significant difference (p<0.05) as compared to non-allergic subjects. Following ConA stimulation apoptosis was significantly enhanced (p<0.05) for all cell populations at all time-points.

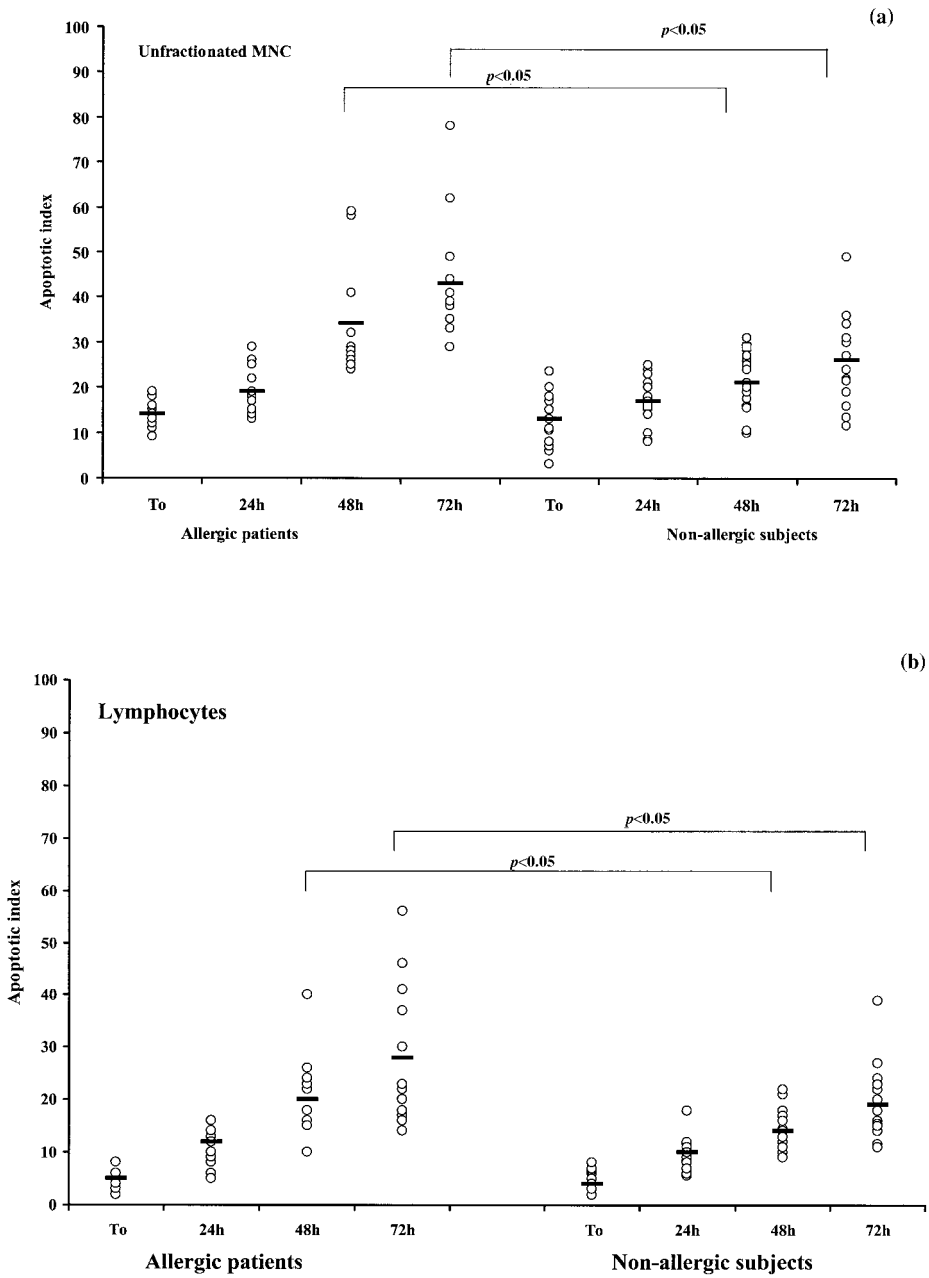

FIG. 2 Spontaneous apoptosis of cells in allergic and non-allergic subjects; (–) mean value; (a) Unfractionated mononuclear cells (MNC); (b) Lymphocytes.

Table 3. Apoptosis of MNC in allergic patients (n = 3) and non-allergic subjects (n = 3) examined by cytofluorymetry with Annexin V-FITC and PI. (AxV- Annexin V, PI- Propidium Iodine, SE- standard error, Sp.- spontaneous).

	Allergic patients			Non-allergic subjects		
	AxV(+)PI(–) Mean ± SE	AxV(+)PI(+) Mean ± SE	Together Mean ± SE	AxV(+)PI(–) Mean ± SE	AxV(+)PI(+) Mean ± SE	Together Mean ± SE
Baseline To	18.1 ± 3.45	2.9 ± 1.1*	21.0 ± 3.8	14.4 ± 5.9	7.7 ± 4.5	22.2 ± 7.9
24 h sp.	27.2 ± 9.0 *	13.3 ± 6.8	40.4 ± 13.4	15.1 ± 1.8	11.5 ± 8.3	26.6 ± 9.2
24h ConA	51.2 ± 9.1	18.8 ± 8.8	70.4 ± 11.9	31.9 ± 5.9	35.9 ± 11.9	68.1 ± 14.5
48 h sp.	27.3 ± 7.7	27.6 ± 16.3	54.7 ± 11.6	19.9 ± 10.4	24.9 ± 19.3	44.6 ± 17.8
48 h ConA	36.8 ± 9.6	39.1 ± 16.2	75.9 ± 9.4	33.8 ± 14.7	34.7 ± 18.4	68.9 ± 17.6
72 h sp.	34.6 ± 9.1	38.3 ± 15.9	77.8 ± 10.6	18.2 ± 5.9	51.5 ± 19.0	72.3 ± 18.7
72 h ConA	34.6 ± 9.1	41.7 ± 15.7	76.2 ± 11.8	26.9 ± 8.8	48.4 ± 18.8	76.0 ± 14.9

* $p < 0.05$; significantly different from non-allergic control.

A significant proportion of unfractioneted mononuclear cells, (both lymphocytes and monocytes) demonstrated apoptosis immediately after isolation (T_0), and after 24, 48 and 72 hours of culture the apoptotic index for all cell populations significantly increased. (Table 2). Apoptotic index of unfractionated MNC and lymphocytes was significantly higher in allergic patients as compared to non-allergic subjects after 48 and 72 hours of culture ($p < 0,05$). Figure 2a,b. The proportion of apoptotic monocytes was not different between atopic and non-atopic subjects at any time.

Incubation of cells with Con A (10 μg/ml) resulted in a significant increase in the proportion of apoptotic cells in all cell populations. The apoptotic index for MNC and lymphocytes were significantly higher in atopic as compared to non-atopic subjects at 24, 48 and 72 hour of culture with ConA.

Flow-cytometric analysis

In order to confirm morphologic observations, apoptosis of MNC was further analyzed in 3 allergic and 3 non-allergic patients by cytofluorymetry. Again a higher proportion of apoptotic cells was detected in allergic as compared to non-allergic subjects. Table 3.

Representative plots for allergic and non-allergic subjects are shown in Figure 3.

DNA fragmentation

The presence of apoptosis was confirmed by DNA ladder. The fragmentation of DNA was observed after 72hrs culture of MNC only in those atopic patients, in whom the percentage of apoptotic cells was higher then 50% (n = 4). No fragmentation of DNA could be

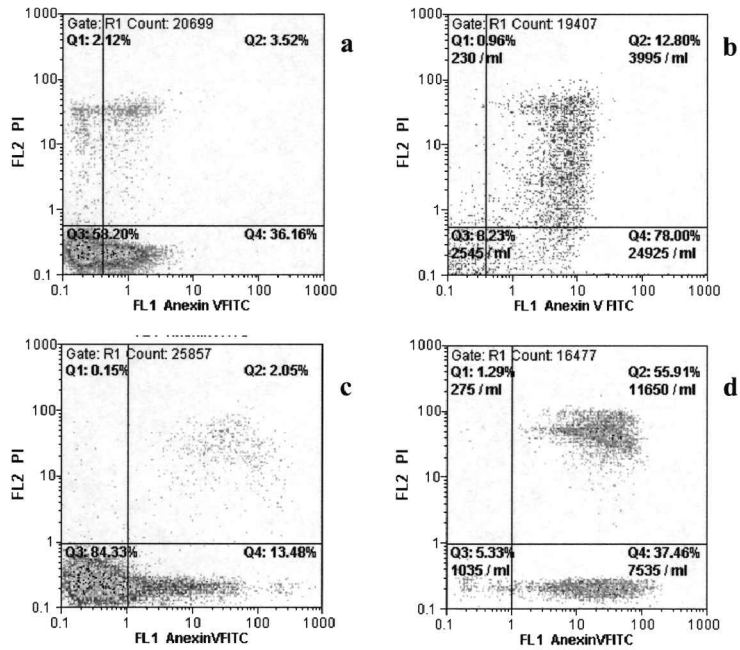

FIG. 3. Representative dotplots for cytofluorymetric method. Spontaneous and ConA stimulated (48h) apoptosis of MNC in an allergic patient (a and b panels); spontaneous and ConA(48h) stimulated apoptosis in non-allergic subject (c and d panels).

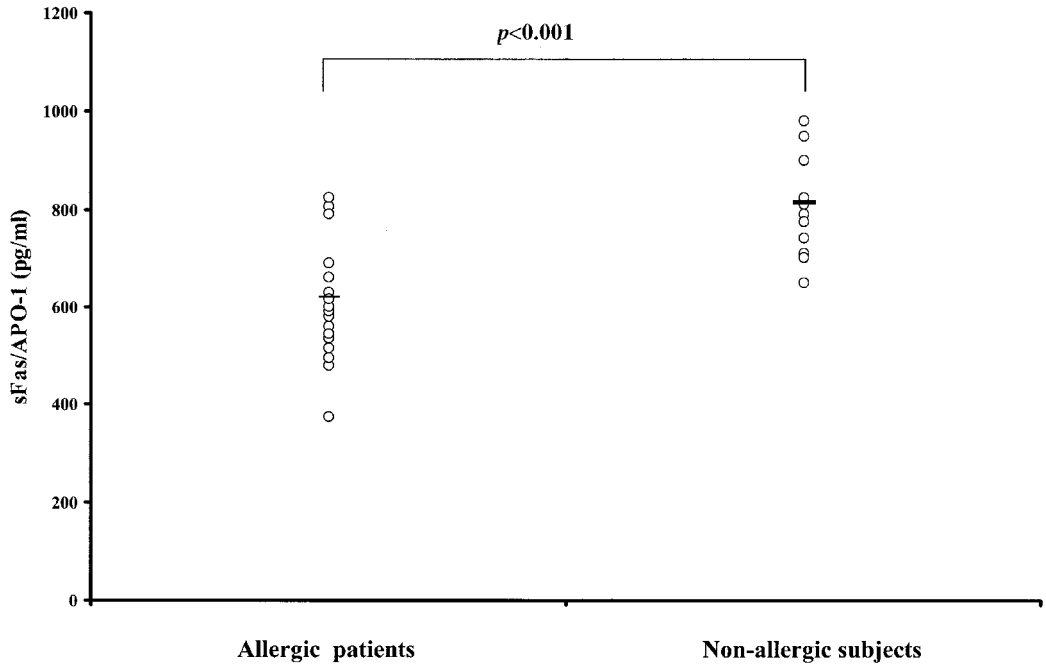

FIG. 4. Serum levels of sFas/APO-1 in an allergic and non-allergic group. (–) mean value.

detested in atopic patients with apoptotic index < 50% (n = 6) or in non-atopic subjects (n = 4).

Serum sFas levels

In allergic patients, mean serum sFas level, was significantly lower then in non-allergic group (mean value 624.8 pg/ml ± 25.67 versus 802.0 pg/ml ± 31.9; p = 0.003). Figure 4. There was an inverse correlation between sFas and baseline (T_0) apoptosis of MNC in both groups (r = –0.12; p < 0.001).

Serum ICE/caspase-1 levels

The mean ICE/caspase-1 concentration was significantly higher in sera of allergic patients as compared to non-atopic group (mean value 27.71 pg/ml ± 3.79 vs. 23.54 pg/ml ± 2.35 respectively; p < 0.01). Figure 5.

Significant relationship between ICE/caspase-1 levels and baseline (T_0) apoptosis of MNC was observed only in atopic asthmatic patients (r = 0.57; p < 0.001). Figure 6.

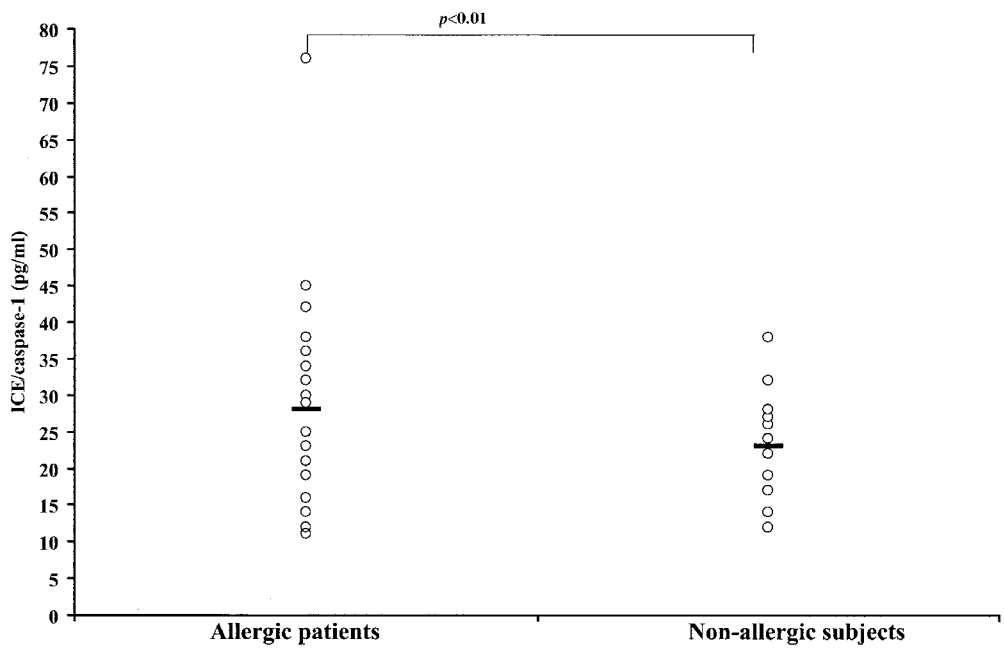

FIG. 5. ICE/caspase-1 levels in serum of allergic patients and non-allergic subjects. (–) mean value

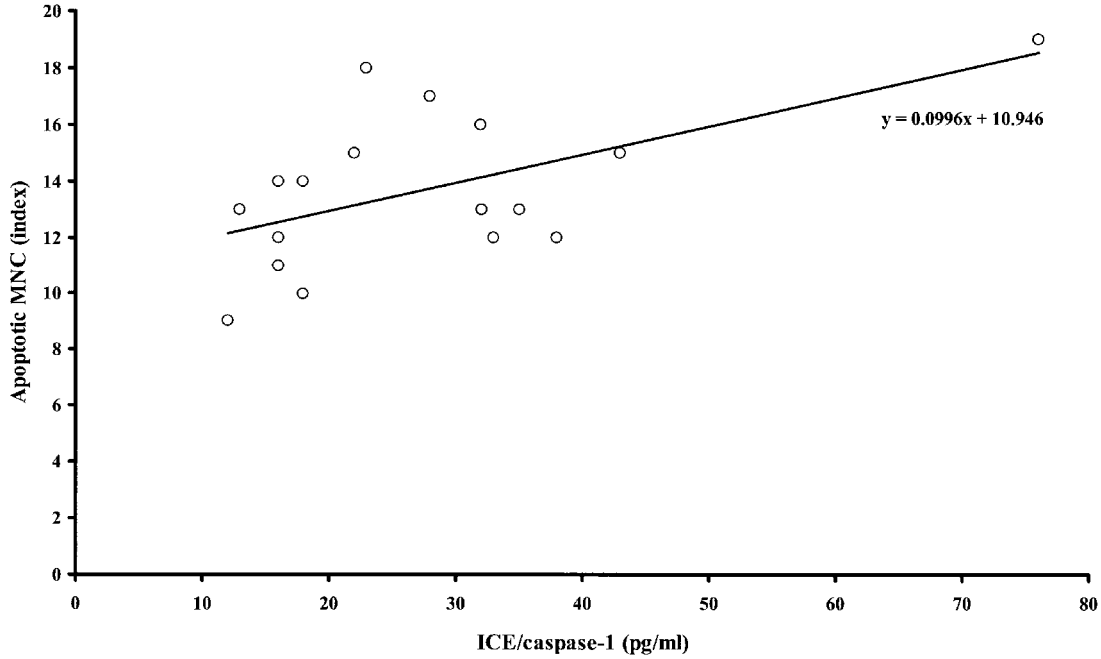

FIG. 6. Relationship between serum ICE/caspase-1 levels and T0 apoptosis of MNC in an allergic patients.

Serum TNF-α levels

There was no significant difference in sera TNF-α concentration between the two-studied group [12.11 pg/ml ± 3.11 (range 0–40 pg/ml) and 7.18 pg/ml ± 2.90 (range 0–26 pg/ml) in allergic and non-allergic subjects respectively]. A positive correlation between baseline apoptosis of MNC and serum levels of TNF-α was found only in non-allergic subjects (r = 0.58; $p < 0.001$).

Discussion

Our study demonstrated, that immediately after isolation a significant proportion of mononuclear cells in peripheral blood of asthmatic patients and healthy subjects undergo apoptosis, and that the percentage of apoptotic cells increases with time of culture. Although the original observation was made with morphologic fluorescent method, DNA ladder and cytofluorymetry/ Annexin method confirmed the presence of apoptosis in mononuclear cells.

Interestingly, we found that a significantly higher percentage of apoptotic mononuclear cells were present in patients with allergic asthma and/or rhinitis as compared to non-allergic subjects. Although patients suffered from perennial rhinitis and/or bronchial asthma, at the time of the study they were in stable conditions and were not taking any oral medication suggesting, that an increased MNC apoptosis in allergic patients was related to their atopic status or/and to inflammatory process in their airways. Moreover we have observed and increased apoptosis of MNC in atopic patients after cell stimulation with mitogen Con A indicating the MNC from allergic patients are also more susceptible to induction of apoptosis as compared to non-atopic MNC. In order to characterize the populations of MNC undergoing increased apoptosis lymphocytes and monocytes (adherent cells) were studied separately. Although both MNC populations demonstrated apoptosis, only apoptosis of lymphocytes was increased in allergic patients.

The significance of our finding for the pathogenesis of allergic airway disease is not clear. The lymphocytes are recruited from the bone marrow to the peripheral blood and then to the site of allergic inflammation in the airway. The airway mucosa lymphocytes generate several cytokines e.g. IL-4, IL-2, IL-5 and IL-13 with proinflammatory properties, thus actively participating in the development of the effector phase of allergic inflammation.[20,21,22,23] Increased apoptosis of lymphocytes in peripheral blood of allergic patients is in contrast to decreased apoptosis observed at the site of inflammation in the bronchial mucosa of asthmatics.[24] However, it is conceivable, that inflammatory mediators (e.g. proteases, including caspase-1) released in the airways may reach cells in peripheral blood resulting in enhancement of their apoptosis. On the other hand the cytokin profile of lymphocytes in the peripheral blood from allergic patients differ from the profile of the healthy persons suggesting that different cell subpopulation at different stage of activation are recruited from bone marrow to the peripheral blood and further into the airways. This cells may differ in their response to apoptotic stimuli (e.g. Caspase-1) present in serum, thus resulting in enhanced apoptosis. Increased apoptosis of lympho-

cytes in peripheral blood could be a regulatory mechanism down regulating further recruitment of mononuclear cells to the site of inflammation.

In order to get some insight into the mechanism of increased apoptosis of allergic patients with atopic allergy, factors related to apoptosis sFas, caspase-1 and TNF-α were measured in serum in parallel with assessment of apoptosis. Fas antigen is a member of the tumor necrosis factor receptor superfamily responsible for controlling of the cell death signal.[25,26] Cell-surface Fas occurs also as a soluble protein sFas which can be detected in serum. In this study, we demonstrated significantly lower serum sFas in allergic asthmatic patients as compared to non-atopic subjects. Our observation is line with previous study of Kato et al.[27] who described lower levels of serum sFas in patients with symptomatic allergic rhinitis as compared to healthy persons. Jayaraman S, et al.[28] observed, that asthmatic subjects had 23% lower levels of Fas + T cells (in the airway mucosa) during treatment with glucocorticoids and suggested that selective resistance to Fas-dependent apoptosis may reflect altered antigen-driven, accessory cell-dependent signaling. Downregulation of Fas mRNA and surface Fas receptor on pulmonary CD3+ T lymphocytes from patients with asthma were also reported.[29] These data suggest that an ineffective activation of Fas signal transduction at airway mucosa may contribute to increased T cell-dependent inflammation in asthma. However, in recent study the INF-γ + but not IL-4+ T cells in the asthmatic biopsies had significantly higher proportions of apoptotic cells compared with the control group.[30] In our study, the number of apoptotic lymphocytes in peripheral blood was increased in allergic patients and sFas concentration demonstrated a weak but statistically significant negative correlation with apoptotic index in both allergic and non-allergic subjects, indicating a possible and unexplained relationship between sFas release and the rate of MNC apoptosis.

Apoptosis is implemented by intracellular activity of a family of cysteine proteases called caspases. Activation of cascade caspases during apoptosis results in the cleavage of critical cellular substrates, including poly (ADP-ribose) polymerase and lamins, so precipitating the dramatic morphological changes typical of apoptosis. ICE/caspase-1 (Interleukin-1β-converting enzyme) was the first member of the caspase family to be identified as a novel type of cysteine protease responsible for the conversion of precursor Interleukin-1 to its mature form and may be a key mediator of inflammation. Our study demonstrated a significant increase in ICE/caspase-1 level in serum from allergic asthmatic patients as compared to control group, and a correlation between baseline apoptosis of MNC and serum ICE/caspase-1 levels in atopic asthmatic patients. The increase in the caspase level may be secondary to increased apoptosis of MNC. Alternatively we cannot exclude that it may be casually related to an increased apoptosis in allergic patients. In addition to FAS antigen TNF-RI receptor has been implicated in the apoptosis of lymphocytes in several diseases including asthma.[31,32] However, we did not find any significant differences in TNF-α serum levels between allergic asthmatic patients and non-allergic subjects, which does not support the role of TNF-α in observed increased apoptosis of lymphocytes in allergic patients. Interestingly, the correlation between apoptosis of MNC and TNF-α concentration was present only in control group that may point to differential regulation of apoptosis in allergic and healthy subjects.

In conclusion, our study demonstrated an increased apoptosis of MNC (presumably lymphocytes) from peripheral blood of atopic asthmatic patients and an increased susceptibility of these cells to mitogen – induced apoptosis. In parallel significant changes in serum levels of sFas and ICE/caspase-1, correlating with apoptosis were observed, suggesting differential regulation of apoptotic process in peripheral blood mononuclear cells of patients with allergic asthma and/or rhinitis.

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