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The Implication of Morphological Characteristics in the Etiology of Allergic Asthma Disease and in Determining the Degree of Severity of Atopic and Bronchial Asthma

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ABSTRACT

Apoptosis of immuno-competent cells involved in controlling the development of atopic and bronchial asthma is a physiological process characterized by specific morphological feature. Therefore, the aim of the present study was to evaluate the morphological changes and their impact on diagnosis of bronchial and atopic asthma, with special emphasis on apoptotic markers of lymphocytes of asthmatic patients according to their degree of severity. In the present study, both morphological and biochemical approaches were used to study the implication of lymphocytes in the pathogenesis of allergic asthma. The morphological study was carried out using optical and electronic microscopes and the rate of DNA fragmentation via the method of flow cytometry and electrophoretic agarose gel. The morphological and DNA fragmentation results obtained showed the deregulation of apoptosis of lymphocytes of asthmatic patients with bronchial and atopic asthma but for every individual patient from each group. The presence of chromatin spotting without the degradation of DNA into fragments of high molecular weight and extensive cytoplasmic swelling and vacuolization in asthmatic patients with serious severity gives the impression of an intermediate cell death phenotype such as aponecrotic-like. Thus, the death of lymphocytes of asthmatic patients with serious severity is related to a specific structural feature that can be described as aponecrotic cell death-like, occurring during the deregulation of apoptosis. It is commonly thought that the subtle changes in the lymphocytes of asthmatic patients may be a direct result of the relative degree of severity of pathology or of a degree of allergen.

Key words: Apoptosis, DNA fragmentation, lymphocytes, morphological feature, bronchial asthma

INTRODUCTION

Bronchial and atopic asthma is a global problem, however, the most significant successes in the etiology of this disease are achieved over the last decade. These acquisitions have found their echo in the report of GINA group (Global Initiative for Asthma) 1996, revised in 2002 (the overall strategy of treatment and prophylaxis of bronchial asthma (2002) and 2006. In this study we note the definition and classification of bronchial and atopic asthma and modern look that we

can have on the etiology and pathogenesis of the disease without overlooking the economic loss of the society, conditioned by this pathological disease. Despite the advanced studies in the field of immunology and pneumology, the functional diagnosis of bronchial and atopic asthma remains a current problem (Ovsyannikov *et al.*, 2007). Several studies on different medicinal means, the means of combinations of the drugs for the treatment of diseases and dosing regimen may help to improve the control of the disease progression (Tsoi *et al.*, 2006). The lack of data on the control of asthma led to the creation of several evaluation systems of control, based on the analysis of clinical and functional data. Therefore, among the basic criteria for diagnosis, it is important to consider the morphological aspects of the disease development that is to search for new molecular markers that may help to diagnose asthma. Thus, Urbonien *et al.* (2005) highlighted the role of apoptosis and autoimmunity in the pathogenesis of chronic respiratory diseases, particularly bronchial and atopic asthma. The pathogenesis of asthma is defined as an inflammatory process in the specific bronchial space in which bronchial eosinophilia, macrophage and T-lymphocytes play a central role (Aubier, 1999; Vignola *et al.*, 2000). In the development of the allergic reaction. The specificity of the inflammatory process of bronchitis at the bronchial wall in asthma is characterized by an increase of immuno-competent cells in the mucosa and within the bronchial tree (Gavalov *et al.*, 2001) followed by a loss of epithelial cells in the bronchi (Trautmann *et al.*, 2000). All this is accompanied by DNA fragmentation, characteristic of a change-specific apoptosis and activation of T cells and eosinophilia (Rottem and Shoenfeld, 2003; Urbonien *et al.*, 2005). Apoptosis of cells characterized by specific morphological feature (Wyllie, 1980; Earnshaw, 1995; McIlroy *et al.*, 2000) is one of the mechanisms being involved in the control of homeostasis of lymphocytes in the course of immune response. According to some authors, the majority of lymphocytes infiltrating the respiratory route during asthma are apoptosis stable which allows assuming that the steadiness of inflammation of the respiratory route depends on the increase in the survival of these cells in the bronchi. But the current data of apoptosis of cells in the tissues or target organs, mainly in the peripheral blood during atopic and allergic diseases is contradictory. For some authors, the programmed death of the majority of T-lymphocytes is related to a selective migration of lymphocytes controlled by antigens (Rottem and Shoenfeld, 2003; Urbonien *et al.*, 2005). For other authors, the persistence of allergens during the development of asthma may lead to an increase in life span of these lymphocytes and slowing down the apoptotic process (Thomas, 1992; Melis *et al.*, 2002; Tsaporova *et al.*, 2003). Some believe that the increase in the number of lymphocytes or their life span is due to deregulation of the apoptotic process.

Druilhe *et al.* (1998) and Vignola *et al.* (1999, 2000) reported that the percentage of apoptotic cells (eosinophilia, macrophages and lymphocytes) of asthmatic patients is less than the number of apoptotic cells in relatively healthy donors with the aid of the method of Tunnel. But they failed to notify the morphological parameters and the potential impact of the severity on the etiology of this disease. Therefore, the objective of the present study was to evaluate the morphological changes and their impact on diagnosis of bronchial and atopic asthma, with special emphasis on apoptotic markers of lymphocytes of asthmatic patients according to their degree of severity.

MATERIALS AND METHODS

Blood sample preparation: The study was carried out on the lymphocytes isolated from peripheral blood of relatively healthy and asthmatic individuals. Forty three patients including 23 men and 20 women with an average age of 31 ± 8 years and 15 control donors who were not

suffering from the bronchial and atopic asthma with an average age of 28 ± 5 years were studied. The group of patient consisted of people of different severity: 16 patients were slightly affected; 9 mild severity and 18 patients were severely affected. The severity of asthma in these patients was assessed according to the Global Initiative for Asthma guideline (National Heart, Blood and Lung Institute, 1995; National Asthma Education and Prevention Program, 1997). The diagnosis of the bronchial and atopic asthma was established on the basis of the data of allergic anamnesis, experiments of nasal provokers and inhalers, results of skin with allergens and dust. All patients and control subjects had not received oral steroids or changed asthma medications in the 2 weeks prior to recruitment for the study. The patients were hospitalized in the detachment of pneumology. The blood was taken from the veins of donors in the morning before their breakfast. Nine milliliter of blood was taken in a specialized tube containing heparin-Na (EUROTUBO, Spain) from each patient and control subject.

Isolation of lymphocytes: Lymphocytes were isolated according to the standard method of zonal centrifugation proposed by Patel *et al.* (1995) with the mixture of ficoll-verograffin ($\rho = 1.077 \text{ g cm}^{-3}$) (Heyfets and Abalkin, 1973; Antoneeva, 2007). Patel method consists of isolating 95% lymphocytes-T and the viability of lymphocytes was determined by the trypan blue exclusion method.

The culture of lymphocytes: The cells obtained (2×10^6) were diluted in 1 mL solution of RPMI-1640 medium in a flat-bottomed plastic plank (Nung), then adding 10% serum of foetal calf as well as 10 μL of L-glutamine ($200 \mu\text{g mL}^{-1}$) (Flow) (Soroka *et al.*, 2007). The cells were cultured and incubated in CO_2 -incubator (5% CO_2) for 1-6 days (Boychuk *et al.*, 2003; Doering *et al.*, 2004).

Morphological study of lymphocytes: The cells obtained after zonal centrifugation were fixed in 2% glutaraldehyde for 1 h, washed and resuspended in 1 mL of 0.01 M phosphate buffer ($\text{pH} = 8$). The cell were post-fixed in 1% buffered osmium tetroxide (OsO_4) (Abdelmeguid *et al.*, 2008) for 1 h followed by dehydration in a graded series of ethanol (30, 40, 60, 70, 96°C) in acetone and propylene oxide and embedded in epoxy resin 810. Cuts were realized with ultra microtome LKB-3 and observed with electron microscope (Hitachi 125-Japon) (Sanders and Wride, 1996), after putting the material in uranyl acetate (Tandler, 1990) and lead citrate (Veneable and Coggeshall, 1965) for the contraction.

Extraction of DNA of lymphocytes: The lymphocytes were placed in 1 mL of fresh solution containing sucrose (5 mM MgCl_2 , 0.01 M Tris-HCl ($\text{pH} 7.6$), 0.1 M $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 mM EGTA, PMSF 1 mM, 1% TPHTOH X-100) (Jrbashyan *et al.*, 2003). The cells in this solution were kept at room temperature for 10 min (Ivashchenko *et al.*, 1999). The samples were centrifuged for 15 min at 3000 rpm and 4°C. The depositing of nucleus was diluted in 400 mL of buffer solution for proteinase K (Itoh *et al.*, 1991). Then, 20 μL of SDS (final concentration 0.5%) and 5 μL of proteinase K (final concentration 250 mg mL^{-1}) were added. DNA was extracted with phenol-chloroform mixture (1:1) (Jrbashyan *et al.*, 2003) and then DNA was precipitated with isopropyl alcohol (Itoh *et al.*, 1991). The concentrations and the frequency of purity were obtained using a spectrophotometer after measuring the absorbance at 260 and 280 nm.

Agarose gel electrophoresis: The same quantity of DNA was placed in agarose gel plug (1%) after adding 2 μL of bromophenol blue. The electrophoresis took place on a platform of $10 \times 11 \times 3$ cm with a voltage of 5 V cm^{-1} for 3 h and 30 min at 20°C in a buffer solution containing $2 \mu\text{g mL}^{-1}$ ethidium bromide and after photographing the gel at ultra-violet (Video system DNA Analyzer).

Determination of DNA fragmentation of lymphocytes: The number of cells being in a stage of apoptotic process was calculated by the rate of cells recorded in the dipodiploidy zone after colouring the cells with the Iodide Propidium (IP) (Nicoletti *et al.*, 1991; Ismail-zade *et al.*, 2005). The cells were put in 70°C of ethanol for an hour, then rinsed in a buffer solution and coloured with IP containing 0.1% of triton x-100 and 0.1% of sodium in darkness for 15 min at ambient temperature. The percentage of cells in the dipodiploidy zone was determined by histogram 2 located at the left of the basic peak which corresponds to diploidy cells.

Data analysis: All analyses were performed using paint, Photoshop and word program.

RESULTS

In many pathological diseases, the number of lymphocytes circulating in peripheral blood is an important diagnostic parameter. Therefore, it is also interesting to study the structure of peripheral blood lymphocytes in patients with bronchial and atopic asthma. Optical microscopy of histological preparations is the most simple and accessible method for the study of lymphocytes from peripheral blood of symptomatic patients in this particular case of asthma. With the aid of optical microscope, compact lymphocytes (7-9 microns), with intense staining of the cytoplasm were observed. These lymphocytes had compact and rounded nuclei with small clarification, sometimes in the shape of grain. The cytoplasm was barely visible around the nucleus. It should be noted that data from the optical microscope did not allow highlighting a significant difference in the morphology of lymphocytes of patients with bronchial and atopic asthma according to their degree of severity. On the other hand, the method of electron microscopy reveals substantial morphological differences in lymphocytes of relatively healthy individuals and patients with bronchial and atopic asthma (Fig. 1).

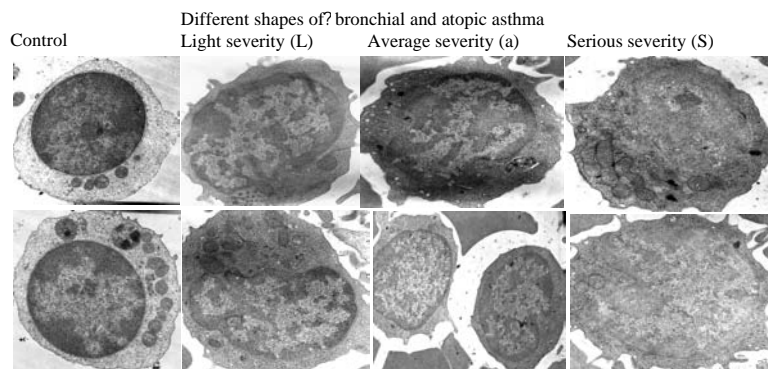


Fig. 1: Morphology of lymphocytes of relatively healthy donors and of atopic and bronchial asthmatic patients with light severity (L), average severity (A) and serious severity (S) *in vivo*

The difference was mainly observed in the nuclei, the structure of the cytoplasm, especially at the cellular components' level. Thus, it was observed that the lymphocytes of asthmatic patients with serious severity were in a state of morphological changes, specific of pathological cells. According to the state of gravity we have the following possible cases:

- **The lymphocytes of relatively healthy donors:** Had a rounded shape (Fig. 2a-c). The cell membrane was well drawn and highlighted without constriction (Fig. 2b). The nucleus, usually large with a rounded shape occupies a large part of the cell. The nucleolus, nucleoplasm and heterochromatin (Fig. 2a-c) were found in the nucleus. Mitochondria, ribosome and granules were observed in the cytoplasm. Microvilli with small nipples (apophysis) were observed in the cytoplasmic membrane of some cells from relatively healthy individuals (Fig. 2b) with peri-nuclear clarification of in some cells (Fig. 2c)
- **The lymphocytes of asthmatic patients with light severity:** Lymphocytes had an oval shape, with apophysis and intussusceptions on the cell membrane (Fig. 3b,c). The nuclei also had an oval shape but most often in improper shape or arranged eccentrically (in relation to the cell membrane) (Fig. 3b,c). The nucleus, important organelle in the cells took a lobed shape with intussusceptions; followed by the collapse of nuclei and its disintegration in micronuclei. But it should be noted that the roughness of the margin of the nucleus is not synonymous with apoptosis of its cells. There was a marginalization and condensation of chromatin in the shape of a half-sphere at the nuclear periphery level (Fig. 3a-c). The structure of the nucleus of some lymphocytes was heterogeneous, with clarification at the chromatin level (Fig. 3a-c). In addition, the components of a cell such as granules and ribosome were located near the nucleus in the cytoplasm (Fig. 3b,c) as if there was a communication between organelles
- **Lymphocytes of patients with average severity:** The lymphocytes had oval shape, with large nuclei showing sometimes intussusceptions, located at the center and occupying almost

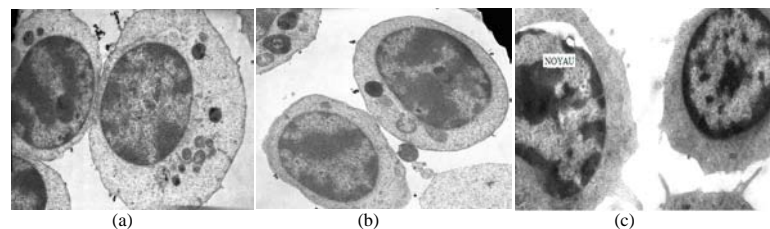


Fig. 2(a-c): Ultra-structure of lymphocytes of relatively healthy donors *in vivo*

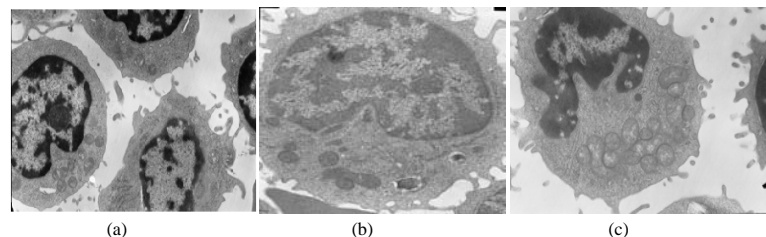


Fig. 3(a-c): Ultra-structure of lymphocytes of asthmatic patients with light severity *in vivo*

the entire cytoplasmic volume (Fig. 4a,b). Unlike the results obtained from asthmatic patients with light severity, there was almost complete absence of microvilli on the surface of cells and a few granules in the cytoplasm (Fig. 4a). Chromatin was observed in the nucleus which showed open and condensed areas

- **Lymphocytes of patients with serious severity:** The ultra-structure of lymphocytes of asthmatic patients with serious severity differs significantly from other groups mentioned above. Lymphocytes had improper shape. Among the ultra-structural and thread like changes, there was alteration in cytolemma and surface texture. First, the loss of microvilli and desmosome in some cells (Fig. 5b) was observed. The swellings and bubbles on the membrane (Fig. 5a,b), known as the blebbing were observed on isolated cells. The structure of cell organelles (mitochondria, endoplasmic reticulum) was not clearly observed. However, there was a large amount of vesicles and granules and large vacuoles (Fig. 5-a,b). There was also a deep cribriform trace in the nuclear membrane, followed by fragmentation of nuclei and there were fragments of the nucleus limited by the membrane inside the cell (Fig. 5). The integrity of the membrane of lymphocytes was often preserved

The analysis of the results obtained *in vivo* showed a significant difference between the morphology of lymphocytes of relatively healthy donors and that of asthmatic patients. The difference was also observed among asthmatic patients according to their degree of severity. This difference was it related to the status of apoptosis of these cells? To answer this question we cultured and incubated the cells isolated from peripheral blood of donors for 3 and 6 days and studied their morphology.

The morphological parameters of lymphocytes of healthy donors after cell culture: At this stage of development, some cells were characterized by the loss of microvilli with a normal wrinkling of cellular membrane. The lymphocytes with a complete change of cell structure were observed (Fig. 6a-d), besides, the air bubbles were found at the plasmatic membrane of cells. In the absence of phagocytosis, it leads to the formation of apoptotic bodies (Fig. 6d) containing condensed or modified organelles. Likewise, the convulsion of the cells usually goes with the condensation of nuclear contents. The aggregation of chromatin and fragmentation of the nucleus follow. Beside these modified cells were found vesicles containing various fragments of cell contents (the residue of chromatin, mitochondria and granules). These vesicles were nothing other than apoptotic bodies formed after the fragmentation of the cell and the nucleus picnotisation during the apoptotic process (Fig. 6-d).

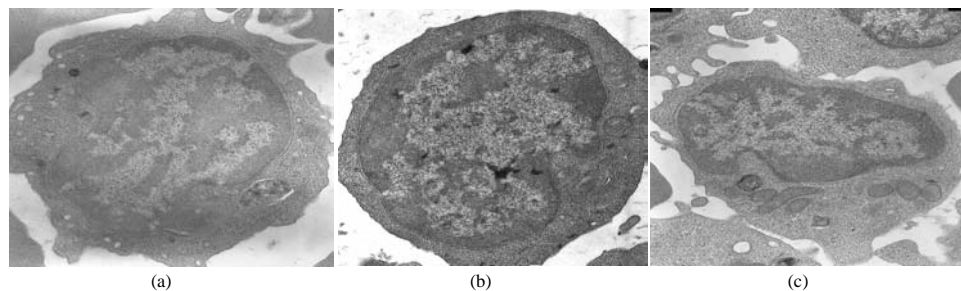


Fig. 4(a-c): Ultra-structure of lymphocytes of asthmatic patients with average severity *in vivo*

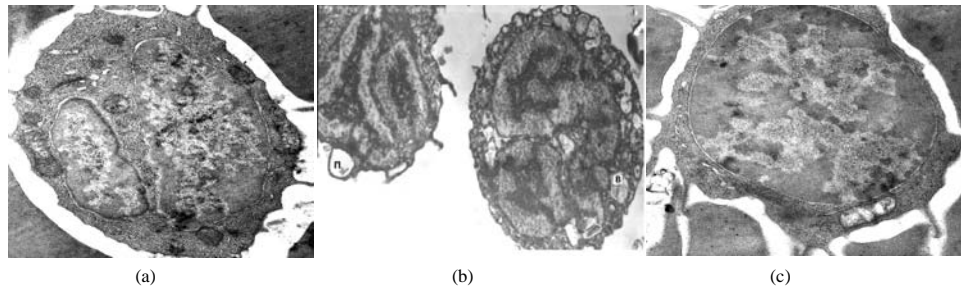


Fig. 5(a-c): Ultra-structure of lymphocytes of asthmatic patients with serious severity *in vivo*

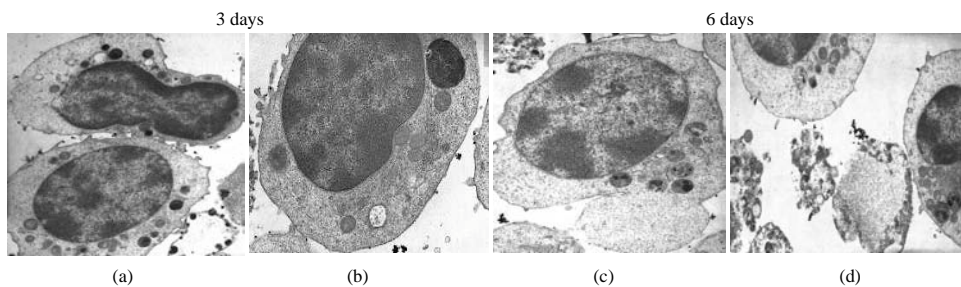


Fig. 6(a-d): Morphological change of relatively healthy donors after 3 to 6 days of culture *in vitro*

After a culture of 3 to 6 days of lymphocytes from asthmatic patients with different severities, structural changes specific to each patient group were observed.

Light severity: After 3-6 days of culture, we observed that some cells have retained structural integrity (Fig. 7) with a regular and visible peri-nuclear space. The cells with a change of nuclear structure were present but likely to divide (Fig. 7b,c). The nucleolus and chromatin condensation (Fig. 7c) were very visible in the nucleus. The cytoplasm generally abundant (opulent), with irregular borders, vary according to the rate of basophilic grains-barely expressed (the cytoplasm discolored) to intense staining of the cytoplasm, with or without peri-nuclear area. Morphological indexes of apoptosis in lymphocytes of patients with light severity were less expressed when compared to the relatively healthy donors.

Average severity: According to the results obtained from electron microscopy after culture of lymphocytes of asthmatic patients with average severity; normal cells, modified cells and proliferative cells (Fig. 8) were identified. After 3 to 6 days of culture, the cells had fair shape with large nuclei centered (Fig. 8a-c) except in some cells (Fig. 8d) where we observe cells in full disintegration with granules and discernable organelles. In the nucleus there was light and condensed chromatin (Fig. 8a-c).

Serious severity: There were no clearly visible membrane structures of cells (Fig. 9a-d), however, a large amount of vesicles and granules were observed. Most importantly, cells with a fragmented nucleus in 2 parts (Fig. 9a), with indistinguishable cytoplasmic (Fig. 9a-c) contents were identified.

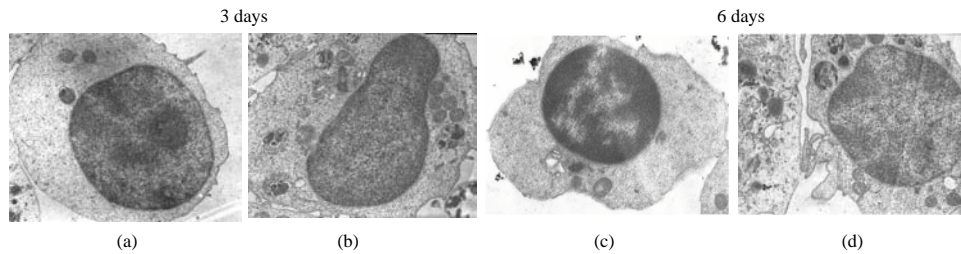


Fig. 7(a-d): Ultra-structure of asthmatic patients with light severity after 3 to 6 days of culture *in vitro*

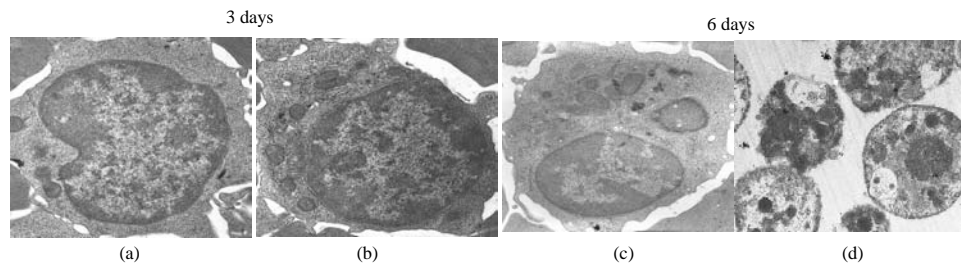


Fig. 8(a-d): Ultra-structure of asthmatic patients with average severity after 3 to 6 days of culture *in vitro*

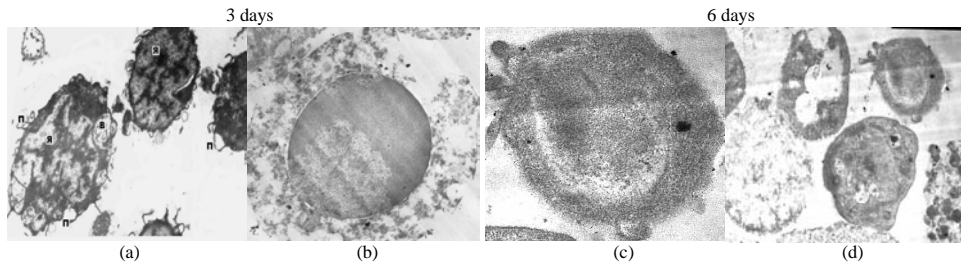


Fig. 9(a-d): Ultra-structure of asthmatic patients with serious severity after 3 to 6 days of culture *in vitro*

Sometimes, in the cytoplasm large and indistinct nuclei (Fig. 9b) were observed, recalling the necrotic cells, large nuclei with intussusceptions occupying almost the entire cytoplasmic space. There were also other cells (Fig. 9d), probably formed as a result of secondary necrosis or superfluous cells that had lost all vitality.

After analysis change in cell structure but was more pronounced in the nuclei of cells according to the degree of severity. Therefore, we studied the rate of the fragmentation of chromosomal DNA according to the degree of severity.

DNA fragmentation of lymphocytes: The characteristics of DNA extracted from lymphocytes of different groups were studied using electrophoresis on 1% agarose gel. For the selection of appropriate conditions of electrophoresis and molecular weight determination of DNA extracted from lymphocytes, standard markers were used: DNA / HindIII (125-564, 2027-2322, 4361-6557, 9416-23,130 bp) (Sibenzym) and 1 Kb Plus DNA Ladder (300, 400, 500, 650, 850, 1000, 1650, 2000, 5000, 12000 bp) (Sibenzim) (Fig. 10). The electropherogram shows (Fig. 10a.) DNA

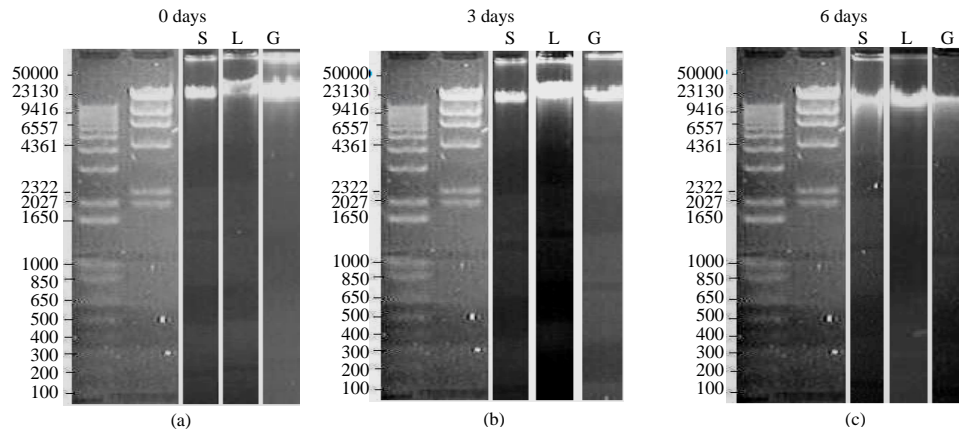


Fig. 10(a-c): Electropherogram showing the degree of DNA degradation of lymphocytes of relatively healthy donors (S) and of asthmatic patients with light severity (L) and serious severity (G) *in vivo* (a) after 3 days (b) 6 days (c) of culture *in vitro*

fragments of 23000 bp for relatively healthy donors. This fragment obtained was it related to apoptosis? To answer this question the degree of DNA fragmentation of lymphocytes during spontaneous apoptosis with electrophoretic method was studied.

The analysis of the products of DNA degradation *in vivo* (before culture) (Fig. 10a) revealed that the DNA extracted from lymphocytes of asthmatic patients with serious severity was the most damaged. A cloud of DNA of different molecular weight between 23000 and 20000 bp was observed. DNA extracted from lymphocytes of patients with slight severity showed fragments of molecular weight between 30,000 and 50,000 bp (Fig. 10a) compared with DNA extracted from lymphocytes of control group (approximately 20,000 to 25,000 bp) (Fig. 10a).

By comparing the product obtained from lymphocytes of the three groups studied (relatively healthy donor (control), asthmatic patients with serious and light severity) (Fig. 10b,c) *in vitro*, we find that incubation of lymphocytes of relatively healthy donors for 3 days causes little pronounced apoptosis with the presence of two peaks (Fig. 10b). The presence of DNA fragments of low molecular weight that varies according to the culture time was observed (Fig. 10b,c). After 6 days of culture the presence of a peak that evolved as a cloud to a molecular weight between 4000-2000 bp (Fig. 10c) was also observed.

The analysis of the degradation products of DNA extracted from lymphocytes of asthmatic patients showed a low rate of inter-nucleosomic degradation of chromatin (Fig. 10). The DNA from lymphocytes of asthmatic patients with light severity was roughly resistant to degradation during the culture for 3 and 6 days (Fig. 10b,c). Based on the results of the electropherogram, it was observed that only the cells from relatively healthy donors were found in advanced level of apoptosis (Fig. 10b,c). The DNA fragmentation did not lead to the formation of fragments of 180-200 bp as reported by some authors.

To raise the ambiguity we compared the rate of apoptotic cells detected in the diploid area of relatively healthy donors and asthmatic patients for 3 and 6 days of culture using the method of flow cytometry.

The quantification of lymphocyte of apoptosis was performed with cytometer using propidium iodide for 3 and 6 days of culture. During apoptosis, DNA degrades with the formation of unordered

fragments. Cell culture for 3 to 6 days leads to fragmentation of DNA of lymphocytes. After 3 days of culture the rate of cells in the diploid area i.e., with a DNA fragmentation was 9%. After 6 days of culture this rate almost doubled (18%). The same trend was observed in asthmatic patients with light severity where the rate increased from 8 to 15%. On the other hand, in asthmatic patients with serious severity, the rate increased from 14 to 21%. But it should be noted a high rate of cells in the diploid area of asthmatic patients with serious severity after 3 days of culture with reference to the results of flow cytometry with propidium iodide. *In vitro*, only a portion of the cells which reached apoptotic stage had 21% in asthmatic patients with serious severity after 6 days of culture.

DISCUSSION

Apoptosis is a highly conserved and integrated response in normal physiological processes of the cell with the morphological and biochemical characteristics and playing a central role in the development of all living organisms (Kerr *et al.*, 1972; Wyllie, 1980; Khorshid and Moshref, 2006). Thus, the possible role of apoptosis in unicellular and multicellular organisms has recently received much attention (Cornillon *et al.*, 1994; Vardi *et al.*, 1999; Frohlich and Madeo, 2000; Lewis, 2000; Ning *et al.*, 2002; Segovia *et al.*, 2003; Bidle and Falkowski, 2004). However, the existence and evolution of apoptosis in unicellular organisms is controversial (Deponte, 2008; Jimenez *et al.*, 2009) and obviously confusing because unlike multicellular organisms, it results in complete loss of the organism (Jimenez *et al.*, 2009). Although, apoptosis is a goal in cancer therapy (Martin and Green, 1994; Houghton, 1999; Sellers and Fisher, 1999; Spierings *et al.*, 2003; Khorshid and Moshref, 2006). The process is characterized by profound morphological change, for instance the condensation of cells and the loss of their contact with neighboring cells and inter-cell matrix. The culture of HL60 cells showed a decrease in cell size anticipating apoptotic features such as translocation of phosphatidylserine on the outside surface of the cell membrane (McCarty *et al.*, 1999; Shirokov, 2007), the release of cytochrome C from mitochondria, the activation of caspase 3 and DNA fragmentation (Maeno *et al.*, 2000). In the early stages of apoptosis, the plasma membrane of dying cells remains intact. The cell sends signals of distress and they were identified and phagocytized by neighboring cells. Among these signals include the exposure of phosphatidylserine on the surface of cell membranes, in addition, the cell membrane has many apophysis called blebbing characteristic of apoptotic cells. The blebbing precede the loss of microvilli and desmosome, the disruption of actin-myosin interaction and the externalization of phosphatidylserine which is expressed by the presence and evaginations of cytoplasmic membrane (Inzhutova *et al.*, 2007). However, the formation of blebbing could not be considered as a specific marker of apoptotic cells but can serve as an integrative parameter indicating the state of progress of the morphological change of cells. The recording of the blebbing on cytoplasmic membrane of lymphocytes can be recommended to clinicians as an expressing method of estimating the endothelial dysfunction (Inzhutova *et al.*, 2007). Electron microscopy is considered by some authors as the most trustworthy and effective means of highlighting this morphological characteristic.

The study of apoptosis of lymphocytes in patients with bronchial and atopic asthma shows the resistance of these cells to this physiological process. It therefore, seems timely the comparative analysis of morphological characteristics of the lymphocytes of relatively healthy donors and asthmatic patients with different severity *in vivo* and *in vitro*. Thus, the ultra-structural differences between the various groups studied were evident. The ultra-structural difference was observed in the nuclei, structure of the cytoplasm and in all cellular components but a particular interest was given to the cell membrane and nuclear structures (Manskokh, 2004). Chromatin was condensed

and arranged in the periphery in the shape of a half sphere. There was deep pressure on the nuclear membrane until the fragmentation of nuclei (Fig. 5a). In some lymphocytes, there was a multiple fragmentation of the nucleus, where chromatin is located in the center (Fig. 5b). At the cell membrane level were observed the absence of microvilli. Sometimes there were cells with disintegrated nuclei and numerous granules. All these changes can hinder the proper functioning of its immuno-competent cells which can have a huge impact on the body of asthmatic patients, especially in patients with serious severity (Fig. 5). At the start of the phenotypic description of apoptosis, it was noted that this process took place in most of the cells and even in all the cells of the living organism. The basis of this assumption is a morphological resemblance of the cells of various tissues via this route. These cells are characterized by a change among which there is a condensation of cells and loss of contact with neighboring cells and the intercellular matrix. In the nucleus, a condensation of the chromatin which usually sticks to the nuclear membrane was observed. This intersecting point of the chromatin and the nuclear envelope is related to a specific functional activity of a portion of the genome. This part of DNA stuck to the nuclear membrane that endured in the first place during apoptosis of lymphocytes. Presumably, this is especially the beginning of the fragmentation of chromatin which is the key event in apoptosis and after its completion, the process becomes irreversible. In the cytoplasm, there are a lot of granules near the nucleus (Fig. 3-5). These changes were considered as annunciator's signs of the progress of the apoptotic process leading to the degradation of DNA. The main manifestation of apoptosis is the enzymatic degradation of chromatin according to literatures. It takes place in a few steps. At the beginning, fragments of 700, 200-250, 50-70 thousand base pairs and fragments of 30-50 thousand base pairs were produced (Rusnak *et al.*, 1996). At this stage, chromatin condensation and intussusceptions of the nuclear membrane were already observed. After the completion of this stage the process becomes irreversible. Then come the next stage of degradation, the disintegration between nucleosomes of DNA that is the rupture of DNA offspring located between nucleosomes which is much more widely studied and used as apoptotic marker. The disintegration of DNA led to the formation of a small fragment of 180-190 base pairs corresponding to base pairs found in one nucleosome.

According to Moskaleva and Severin (2006), in course of caspase-independent mitochondrial pathway, the flavoprotein (AIF) was released from the mitochondria, translocated into the nucleus of apoptotic cells and induced in cooperation with endonucleases (endonuclease G), the cleavage of DNA in fragments of high molecular weight and chromatin condensation. But that chromatin condensation does not lead to the formation of oligonucleotides that can be observed in the caspase-dependent apoptotic pathways (Susin *et al.*, 2000; Moskaleva and Severin, 2006). The analysis of the products of disintegration of DNA *in vivo* (before culture) shows that DNA extracted from lymphocytes of asthmatic patients with serious severity was much more degraded with fragments of high molecular weight DNA extracted from lymphocytes of asthmatic patients with light severity revealed fragments of molecular weight varying between 50000-30000 bp compared with DNA extracted from lymphocytes of control groups ranging from 25.000 to 20.000 bp. Obtaining DNA fragments of high molecular weight can provide information on capcase-independent mitochondrial pathway of apoptosis of lymphocytes of asthmatic patients. It is generally accepted that multiple forms of programmed cell death exist (Manskikh, 2007) and that some of them do not require the activation of caspase (Leist and Jaattela, 2001; Clarke, 2002). Or the obtainment of the fragments may lead us to think probably of a dysfunction of the enzyme capable of causing DNA fragmentation into segments of low molecular weight. What is interesting is that the dying cells

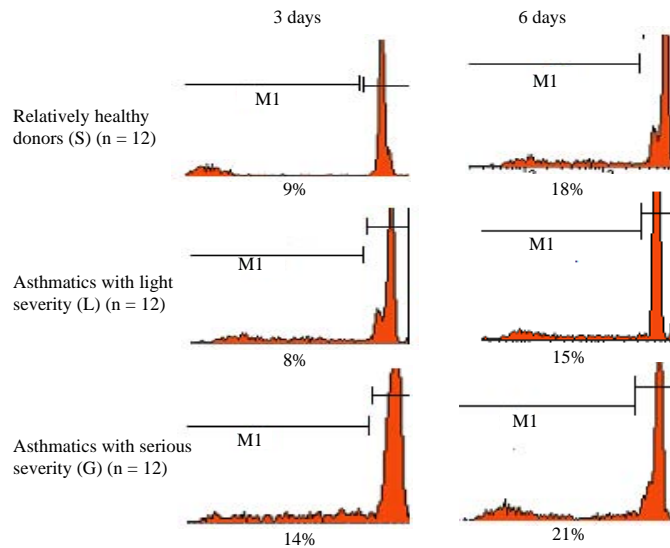


Fig. 11: Cytofluorogramme showing the average percentage of degradation of DNA of lymphocytes of relatively healthy donors (S) and of asthmatic patients with light severity (L) and serious severity (G) with (% counts of cells with a fragmentation of DNA)

retain the integrity of their membranes to the final phase (Bar, 1996; Shirokov, 2007). Brown *et al.* (1993) reported that fragments of high molecular weight showed two peaks in the range of 50 to 300 kbp after separation of DNA of apoptotic cells in the electrophoretic field. The analysis of fragmentation products of DNA extracted from lymphocytes of asthmatic patients showed a low level of inter-nucleosomal degradation of chromatin. The DNA of lymphocytes of asthmatic patients with light severity was resistant to degradation during the culture for 3 and 6 days. As our results have shown on the study of spontaneous apoptosis, only relatively healthy donor cells were found at an advanced level of apoptosis. Therefore, we can assume that fragments from the degradation of DNA of the lymphocytes from asthmatic patients and, especially asthmatic patients with serious severity, were not products of the classical apoptotic process (Fig. 11). Cell culture for 3-6 days leads to fragmentation of the DNA of lymphocytes. Thus, the DNA fragmentation is one of the main biochemical features of apoptosis which takes place in the nucleus of cells. Not all the cells that reached the apoptotic stage after staining with propidium iodide. Similarly, when compared to cells of healthy individuals, only a portion of lymphocytes of asthmatic patients *in vitro* was exposed to spontaneous apoptosis. The cells of patients with serious severity exposed to the terminal phase of apoptosis were 14-21%. Referring to Woolcock (1993), Chan *et al.* (1998) and Pinegin (2000) reports, we could conclude that the peripheral blood lymphocytes of asthmatic patients with serious severity show destructive changes, characterized by asynchronous development of the nucleus and cytoplasm. *in vitro* we observed that the lymphocytes of asthmatic patients with serious severity showed no major change leading to the formation of apoptotic bodies. In this present study, both morphological and biochemical were used to study the particularity of lymphocytes of asthmatic before death. Our results showed the presence of chromatin spotting without DNA fragmentation and extensive cytoplasmic swelling and vacuolization in asthmatic patients with serious severity. This appearance gives the impression of an intermediate cell death phenotype such as aponecrotic-like. Thus, the death of lymphocytes of asthmatic patients with

serious severity is related to a specific structural feature that can be described as apoptotic cell death-like, occurring during the deregulation of apoptosis, by referring to the results of Jimenez *et al.* (2009). According to the recent study of some authors, apoptosis is not the only way by which cell may die (Manskikh, 2007). Evidence shows that other alternative forms of non apoptotic exist in parallel with apoptosis (Golstein and Kroemer, 2005; Bredesen, 2007). It is commonly thought that subtle or dramatic changes in the cell death phenotype may be a direct result of the relative degree of severity of pathology or of allergen. The morphological and DNA fragmentation results obtained showed the deregulation of apoptosis of lymphocytes of patients with bronchial and atopic asthma but for every individual patient from each group.

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