

Nuclease Activity Associated with Secreting Granules by Lymphocytes in Patients with Bronchial Asthma

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Received date: September 30, 2015; Accepted date: December 14, 2015; Published date: December 21, 2015

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Abstract

Background: We know, through recent studies, the existence of some morpho-biochemical peculiarities in the process of type 1 programmed death of patients' lymphocytes suffering from bronchial asthma, but little convincing data exist on the activity of enzymes involved in this physiological process. Therefore, the aim of our research was to study the enzymatic activity of secreting granules of patients' lymphocytes with bronchial asthma, according to the degree of severity.

Method: The study was based on the role of granular extracts in the process of programmed death isolated lymphocytes from peripheral blood of relatively healthy individuals and asthmatic patients with different severity. The immunological characteristics of lymphocytes was done with the radial immune-diffusion method and ELISA test but the method of agarose gel electrophoresis help us to detect the catalytic activity of protein extracts of secreting granules of lymphocytes.

Results: The results obtained showed that lymphocytes from asthmatic patients with severe severity are characterized by a decrease in cytotoxic T lymphocytes content balanced by an increase in T-Helper lymphocytes. We also noticed the enzymatic activity at all the groups studied but this activity was relatively high in asthmatics with severe severity. Furthermore, the study of the cationic dependence has allowed to establish an increase in enzymatic activity in all the groups studied after incubation of DNA in a medium containing Ca^{2+} with a pH of 7.5 unlike ions Mn^{2+} which seem to reduce the enzymatic activity. The expression of enzymatic activity in the presence of zinc allows us to suggest the presence of DNase acid in granules, which activity is not necessarily associated with divalent metal ions.

Conclusion: Based on the above results, one might conclude that the secreting granules have a high enzymatic activity but with a strong cationic dependence. This not only allows a better understanding of the morphological changes observed during the course of apoptosis in lymphocytes of patients but also brings more to the knowledge of the enzymatic influence in the process of type 1 programmed death.

Keywords: Granule; Apoptosis; Lymphocytes; Bronchial asthma

Introduction

The findings of recent years suggests the possibility of a common pathogenic pathway in the course of the development of atopy, allergy and autoimmunity, as these processes are interrelated by a perturbation of the functioning of immune system where lymphocytes play a central role [1-4]. These lymphocytes, although playing an important role in the immune response, are controlled by the process of programmed cell death (PCD) of type 1 [5-8]. Many researchers, including Zhivotovsky et al. [9,10] believe that the PCD of type 1 falls due owing to the enzymatic degradation of deoxyribonucleic acid in the cell nucleus. Thus, the DNA fragmentation is one of the most important biochemical signs in the process PCD of type 1 [11].

However, the role played by these enzymes involved in this process and their mechanism of action is misunderstood. The current data confirm the role of certain nucleases in PCD. Thus, some authors believe that the endonuclease G released from mitochondria initiates DNA fragmentation and thereafter, this process of fragmentation is increased by DNase I released from necrotic cells. This process had its scope in necrosis of oncogenous hepatocytes [12,13]. Oliveri et al. first demonstrated in vivo that the enzyme activity of DNase (Ca, Mg-dependent) emerges in the cells exposed to apoptosis induced by drugs, and that performs the degradation of DNA at nucleosome level in human cells [14]. Lipskaya [15] and Meng et al. have shown activation of DNase (Ca, Mg-dependent), which is inhibited in the presence of zinc ions in the process PCD of type 1 [16]. Likewise, it was discovered another nuclease activity associated with secreting granules by cytotoxic monocytes of CD14+ and CD16+ phenotypes in

patients with autoimmune diseases such as severe diseases and multiple sclerosis [17]. This DNase is more active on double-stranded DNA than on single-stranded DNA and differs from the other three types of endonucleases that are involved in DNA fragmentation (DNase I, DNase II, and NUC 18) [18]. According to author's, this nuclease is involved in the mechanism of programmed death or lysis of cytotoxic T lymphocytes and may be associated with a change of the autoimmune response that depends on T lymphocytes inducing apoptosis. So far, there is no conclusive data on the nature and role of nucleases involved in the process of programmed death of lymphocytes in asthmatic patients. Because of the ambiguity of the information available on the subject, we decided to study the enzymatic activity of secreting granules of lymphocytes in patients with bronchial asthma according to the degree of severity.

Materials and Methods

Patients and blood sampling

The study was carried out on the lymphocytes isolated from peripheral blood of relatively healthy donor and asthmatic individuals. Forty eight patients with an average age of 31 ± 8 years and 15 control donors who were not suffering from the bronchial asthma with an average age of 28 ± 5 years were studied. The group of patient consisted of people of different severity: 10 intermittents, 13 patients were mildly affected, 10 with moderate severity and 15 patients were severely affected. The severity of asthma in these patients was assessed according to the Global Initiative for Asthma guideline [19,20]. The diagnosis of the bronchial asthma was established on the basis of the data of allergic anamnesis, results of cutaneous experiments of skin with allergens and dust. All patients and controls had no other chronic diseases. All individuals were non-smokers, did not receive corticosteroids in the 2 weeks prior to recruitment for the study and were selected after informed oral consent had been obtained. All the patients had a history of intermittent chest tightness, wheezing or shortness of breath for at least 5 years prior to study participation consistent with the diagnosis of asthma according to Global Initiative for Asthma. None of the individuals had any respiratory infections in the month previous to their inclusion in the study. No other clinically relevant diseases were reported. The blood was taken from the veins of donors in the morning before their breakfast. Eight ml of peripheral blood was taken in a specialized tube containing heparin-Na (EUROTUBO, Spain) from each patient and control subject. The work was performed in accordance with the rules of the Ethics Committee in the laboratory of Clinical Immunology and Allergy of RKB and with the regulations of the Ministry of Health of the Russian Federation in compliance with the Helsinki Declaration.

Isolation of lymphocytes

Peripheral blood lymphocytes were isolated by the method of zonal centrifugation on a density gradient (3000 rpm/min, K-24) ficoll-verografine ($\rho=1.077$ g/cm) [21,22]. Lymphocyte suspension was washed and collected after centrifugation at 2000 rpm/min for 10 min at 20°C. This method was used to isolate at least 95% of T- lymphocytes according to the data from Patel et al. [23]. The viability of lymphocytes was determined by the trypan blue exclusion method [24-26]. To obtain a pure population of T lymphocytes, we used the negative isolation method with the use of super paramagnetic beads

CD3 (Dynabeads CD3, Dynal, Invitrogen) [27]. The cell populations were analyzed with the flow cytometer FACSCalibur (Becton Dickinson, CIIIA) with the use of Cell Quest and MultiSet software (Becton Dickinson). The cell staining was performed with a dual combination of four colors monoclonal antibody in form of Multitest IMK kit (Becton Dickinson): CD3+; CD8+; CD45+; И CD4+CD3+; CD16+; CD56+; CD45+; CD19+ in accordance with the protocol proposed by the manufacturer. The dosage of immunoglobulin in serum was done with the radial immune-diffusion method and ELISA test was used to determine the levels of IgE.

Obtaining of secreting granules

The secreting granules were isolated from lymphocytes by the method proposed by Podack et al. [28] on gradient of percoll density. For the preservation of the structure of lymphocytes and the obtaining of secreting granules, the lymphocytes were rinsed in 0.32 M of sucrose solution in a buffer solution prepared from 0.01 M Tris-HCl (pH=7.2) and containing 0.003 M of CaCl_2 . After centrifugation at 5000 rpm / min for 20 min, 1 ml of buffer solution of 0.01 M Tris-HCl at pH 7.3 was added to the pellet obtained. Suspend the cells and transfer them to a glass tube of Potter. Grinding the cells in the homogenizers with piston of Potter for 2 min in an ice bath, then add 3 ml of buffer of 0.01 M Tris-HCl solution and incubated for 30 min in an ice bath. Centrifuging the homogenate for 15 min at 2300 rpm/min and at 4°C. Collect the supernatant and wash the pellet with a buffer solution of 0.01 M Tris-buffer and centrifuge again for 15 min at 2300 rpm/min (K-24). Collect again the supernatant. Once again the procedure is repeated, after which the supernatants were gathered for eventual extraction of the granules. The granules was obtained according to Percoll density gradient ($\rho=1.080$ g/cm). Five (5) ml of the supernatant obtained was deposited on Percoll gradient and centrifuged at 19,500 rpm/min for 35 min with the Beckman centrifuge. After centrifugation we recovered on 2 ml 5-7 fractions. For the elimination of Percoll gradient these fractions were centrifuged at 45000 rpm/min for 2.5 hours at 4°C. There was formation of a whitish supernatant containing secreting granules.

Revelation of the catalytic activity of granule extracts

The granules of lymphocytes of asthmatic donors and non-asthmatics were incubated with DNA for 12 hours at 37°C. Take aliquots and add a mixture containing 10% SDS-Na, 0.1% BPB, 50% glycerin to stop the reaction. Allow these aliquots to migrate on a 1% agarose gel for 3 hours at a voltage of 5V / cm. The results of the reaction were recorded using a video system "DNA Analyzer" (НИФ "Литех", Russia).

Data analyses

For quantitative and qualitative analysis of the activity of granule extracts, the gels were scanned and the results were processed with the "Scion image" program [29]. The fluorescence intensity of each area was compared with the total fluorescence intensity for each path considered as 100%. This gives the possibility to define fragmentation index (FI) of DNA for each test and compare the nuclease activity of the different groups studied. In each case the results were compared with a blank as a control where DNA was incubated without protein extracts. We also used the excel package to analyze the data obtained.

Results

Immunological particularity of lymphocytes

T lymphocytes play an undeniable role in the pathogenesis of bronchial asthma because of the importance of their number. One reason for the extension of the performance of this sub-population of cells is their stability to the process of programmed cell death of type 1. Therefore, we analyzed the level of T lymphocyte in the peripheral blood of donors studied. The comparative analysis of the results showed no significant difference in the level of T lymphocyte of the groups studied (Figure 1). According to the literature, T lymphocytes are divided into auxiliary T lymphocytes and cytotoxic T lymphocytes. The analysis of the level of T lymphocytes' subpopulations showed that the group patients with serious severity is characterized by a decrease in cytotoxic T lymphocytes content balanced by an increase in T-Helper lymphocytes (Figure 1).

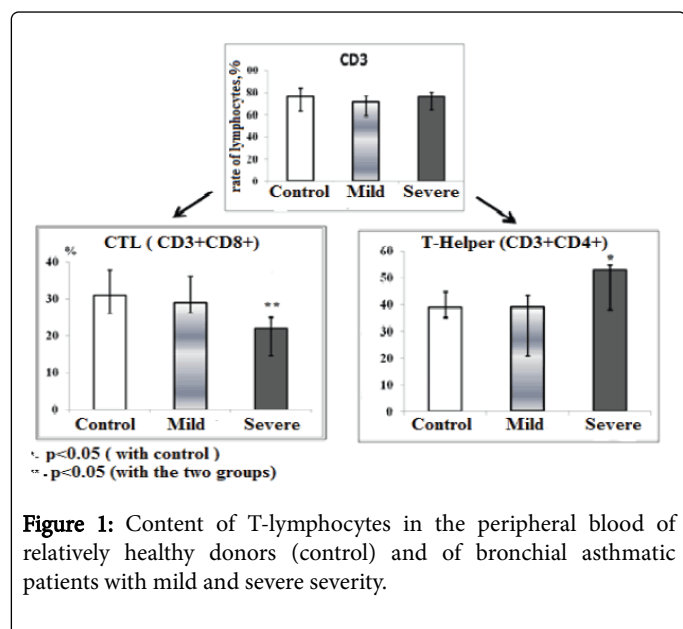


Figure 1: Content of T-lymphocytes in the peripheral blood of relatively healthy donors (control) and of bronchial asthmatic patients with mild and severe severity.

The increase in auxiliary T lymphocytes leads to an increase of the B lymphocytes which differentiate into plasmocytes capable of secreting immunoglobulin (Figure 2). The analysis of the immunoglobulin revealed a significant increase in IgE levels, which would indicate a high probability of the path of allergic process. The increase in the level of T CD4 lymphocytes and B lymphocytes in the group of patients with serious severity (Figure 2) suggests the involvement of humoral adaptive immunity in the pathogenesis of the disease. As any change in functional status is reflected on cell morphology we then set an objective for the next stage, the study of the catalytic activity of granular extracts of lymphocytes of different patient groups selected.

Catalytic activity of granule secreted

The secreting granules extracted from lymphocytes were incubated with the genomic DNA of Hedgehog Sea (50 µg/ml) in 20 mM of buffer solution Tris-HCl at pH 7.5 containing 50 mM Mg²⁺ for 12 hours. Therefore, the presence of the enzymatic activity of protein

extracts in samples of relatively healthy donors and patients with bronchial asthma according to their degree of severity was established. Figure 3 shows the electrophoregram of DNA and the graph illustrating the variation in the enzymatic activity of the different groups studied.

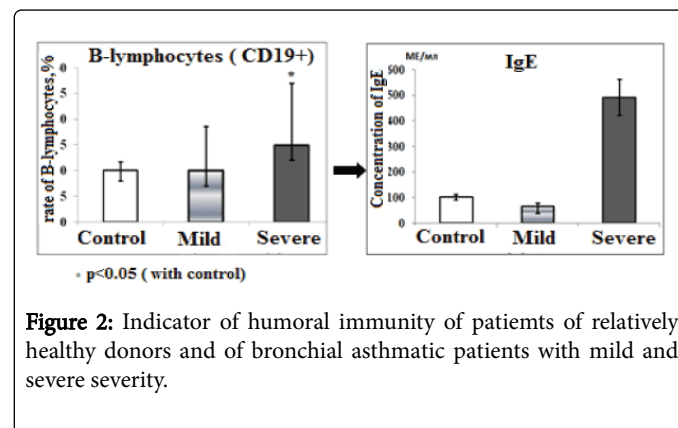


Figure 2: Indicator of humoral immunity of patients of relatively healthy donors and of bronchial asthmatic patients with mild and severe severity.

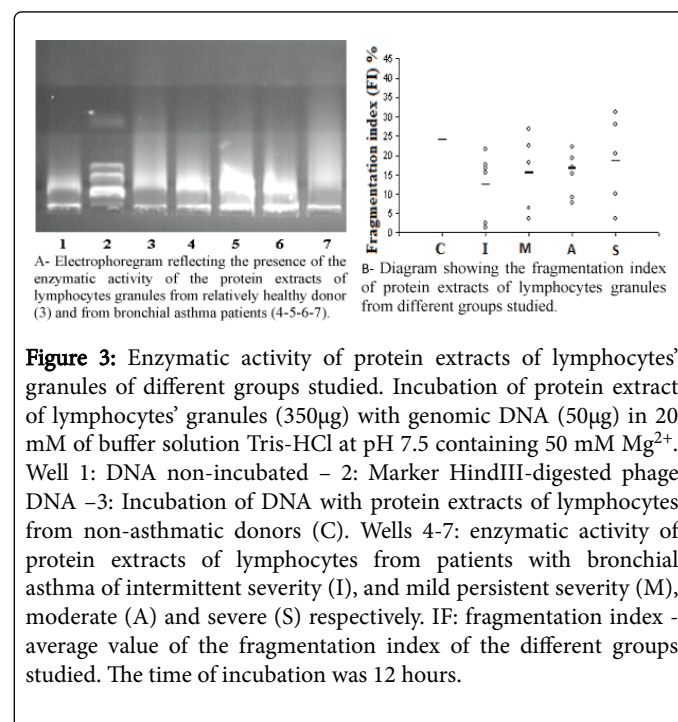
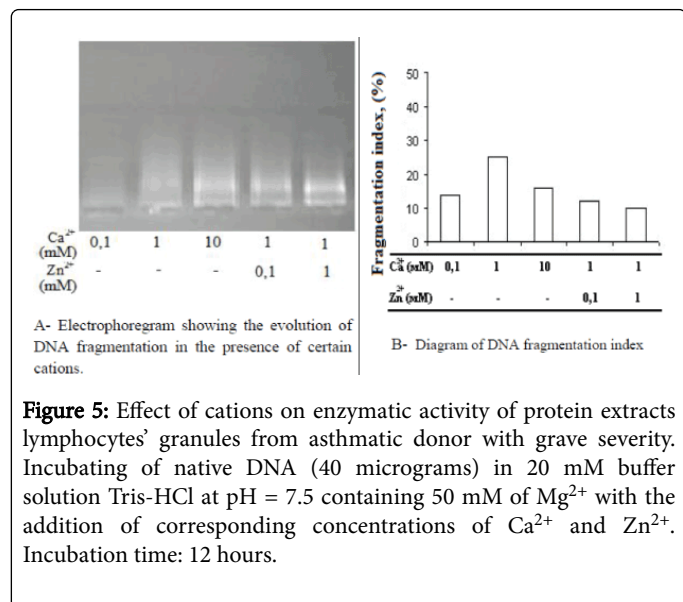
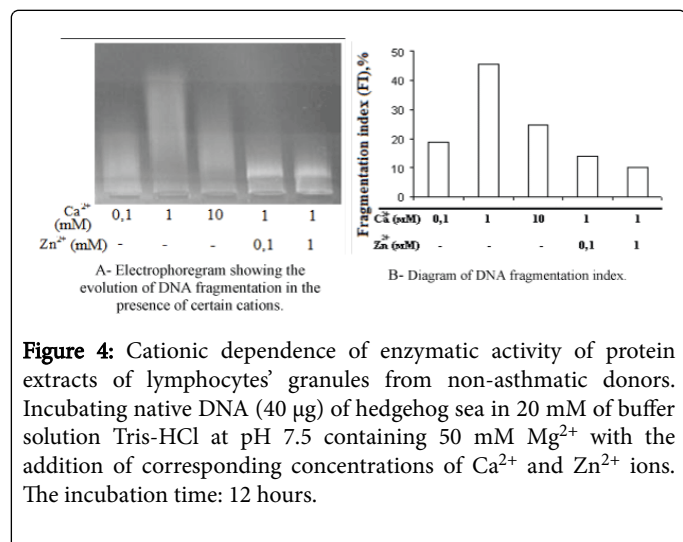


Figure 3: Enzymatic activity of protein extracts of lymphocytes' granules of different groups studied. Incubation of protein extract of lymphocytes' granules (350µg) with genomic DNA (50µg) in 20 mM of buffer solution Tris-HCl at pH 7.5 containing 50 mM Mg²⁺. Well 1: DNA non-incubated - 2: Marker HindIII-digested phage DNA -3: Incubation of DNA with protein extracts of lymphocytes from non-asthmatic donors (C). Wells 4-7: enzymatic activity of protein extracts of lymphocytes from patients with bronchial asthma of intermittent severity (I), and mild persistent severity (M), moderate (A) and severe (S) respectively. IF: fragmentation index - average value of the fragmentation index of the different groups studied. The time of incubation was 12 hours.

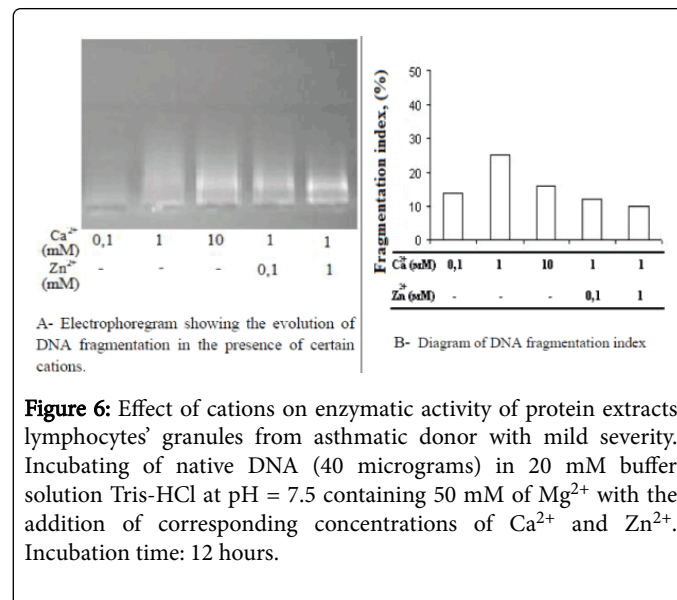
High enzymatic activity of secreting granules of lymphocytes in all groups studied was observed, but this activity was relatively high in healthy donors and asthmatics with grave severity. The individual parameters were characterized by the dispersion (Figure 3b) and probably reflect the immune status of each patient. From the results obtained, a difference in activity between the granular extracts of lymphocyte from relatively healthy donors and asthmatics was recorded in the presence of Mg²⁺ ions considered as an activator of enzymes especially nucleases. This difference was also observed when taking into account the degree of severity. Does the enzymatic activity of these granules depend upon the presence of Mg²⁺ ions? To answer this question we studied the behavior of the granular extracts of lymphocytes from patients, i.e. the identification of the cationic

dependence, the influence of inhibitors (Zn^{2+}) and pH of the medium. We noticed an increase of the enzymatic activity at all the groups studied after incubation of the DNA in a medium containing Ca^{2+} and at a pH of 7.5 (Figures 4-6). This experiment was repeated several times and in all cases there was a high degree of enzymatic activity after adding 1 mM Ca^{2+} in the reactional medium and this activity decreases as the concentration of 10 mM Ca^{2+} increased as well in non-asthmatic donors (Figure 4) as in patients with grave severity (Figure 5) and mild severity (Figure 6).

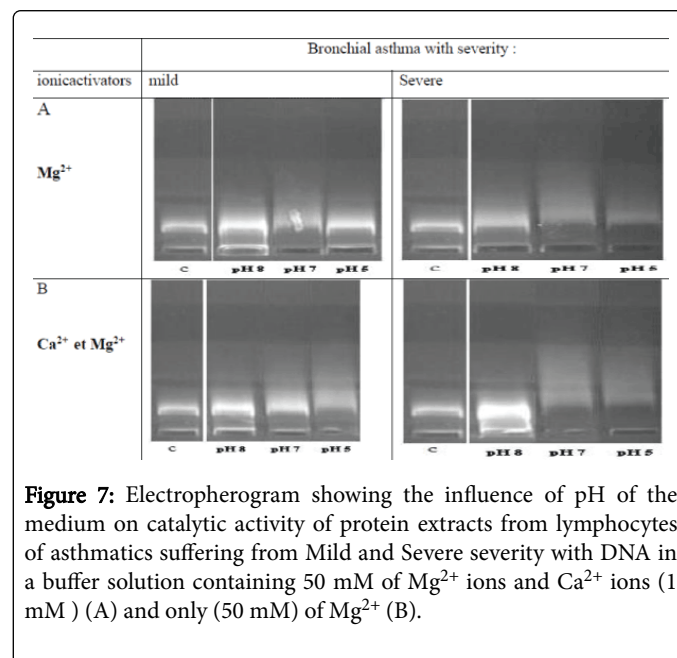


The results obtained show that fragmentation index of granular extracts in healthy donors (Figure 4B) was higher than in asthmatic patients with grave severity (Figure 5B) and mild (Figure 6B). The addition of 1 mM of Zn^{2+} led to the inhibition of the enzymatic activity of granular extracts of lymphocytes by 73% in non-asthmatics (Figure 4B) and 45% in asthmatics with grave severity (Figure 5B) and about 50% in asthmatics of mild severity (Figure 6B). Thus, it should be noted that Zn^{2+} ions had an inhibitory action on granular extracts

of lymphocytes of different groups studied, but it was low in asthmatics (Figures 5 and 6) compared with that observed in non-asthmatics (Figure 4).



To study the pH influence of the medium, we carried out a series of experiments on granular extracted from peripheral blood lymphocytes of patients with mild and grave severity (Figure 7) by varying the pH of the medium (pH.8, pH.7, pH.6). DNA fragmentation was determined in pH 8.0, 7.0, and 6.0 in the presence of Mg^{2+} only (Figure 7A) and Mg^{2+} and Ca^{2+} ions (Figure 7B).



For each test we quantified the degree of enzymatic activity by the fragmentation index (Figure 8).

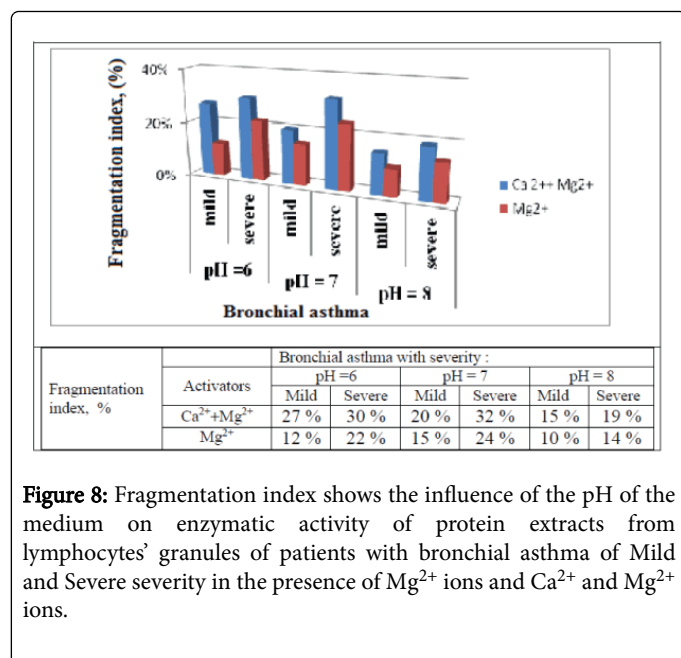


Figure 8: Fragmentation index shows the influence of the pH of the medium on enzymatic activity of protein extracts from lymphocytes' granules of patients with bronchial asthma of Mild and Severe severity in the presence of Mg²⁺ ions and Ca²⁺ and Mg²⁺ ions.

Protein extracts of granular activity was high among different groups studied at acidic pH of the medium in the presence of Ca²⁺ and Mg²⁺ and in the presence of Mg²⁺ only that a high enzymatic activity at a pH of 7.0 especially in asthmatics with severe severity was observed. At pH of 6 and after addition of Ca²⁺ in the medium there was an increase of enzymatic activity in asthmatics of severe severity based on the acidity of the medium, but the fragmentation index reached its maximum at pH 7.0. It is not excluded that this activity is related to the manifestation of other forms of nuclease activity, that is why we studied the enzymatic activities of these protein extracts in the presence of Mn²⁺ ions (25 mM), Ca²⁺ ions (1 mM) in a buffer solution of 20 mM Tris-HCl-buffer at pH 7.5. As shown in Table 1, in the presence of Mn²⁺ ions the enzymatic activity of granular extracts was low, however, there was synergistic action which was several times higher after an additional Ca²⁺.

Degree of severity€	Ions activators			
	Mn ²⁺	Mg ²⁺	Mn ²⁺ +Ca ²⁺	Mg ²⁺ +Ca ²⁺
	Fragmentation Index (FI, %)			
Mild	12,5 %	16,0 %	27,7 %	19,4 %
Severe	20,3 %	23,5 %	41,7 %	31,4 %
Variation of F.I.% De:	62,4%	46,0 %	50,5 %	61,0 %

Table 1: Fragmentation Index in % showing the variation in the catalytic activity of the extracts of lymphocytes' granules from patients with bronchial asthma of mild and severe severity in synergy with some ions.

The addition of Ca²⁺ to the incubation medium in the presence of Mg²⁺ ions causes an increase of enzymatic activity both in asthmatics of mild severity (22%) and grave severity (33.6%). On the other hand, the addition of Ca²⁺ to the incubation medium containing Mn²⁺ ions causes an increase of 121% in asthmatics of mild severity and 105% in asthmatics of grave severity. From the results, we can assume that

during the development of disease severity there was an increase in granular activity of Mn²⁺-dependent (fragmentation index increased by 62.4%) and Ca²⁺ and Mg²⁺- dependent (FI= 61%). After studying the behavior of granular extracts of lymphocytes from asthmatics donors and non-asthmatics on the genomic DNA of the sea hedgehog we underlined the sensitivity of the granular extracts of plasmid DNA (Figure 9) which has the particularity to present itself in a linear, circular and super-coiled shape.

Granular extracts of patients substantially hydrolyze DNA to obtain plasmid DNA in a linear shape through the circular shape (Figure 7). And after 2 hours after the start of the reaction, the rate of plasmid DNA did not increase in a linear shape substantially compared to the amount of linear DNA obtained after one hour of incubation, but there is a more stability of activity in the presence of Zn²⁺ ions. The enzymatic activity is more pronounced in asthmatic of severe severity by referring to the percentage rate of DNA obtained. During our work with granular extracts of lymphocyte there was a case of the expression of protein activity of the extracts in the presence of Zn²⁺ ions where Zn²⁺ ions behaved rather as activators of endogenous activity of nucleases. The use of pBR322 plasmid DNA as a substratum showed that the extract of granules in patients exhibits nuclease activity which is specific for cleavage of single-strands, since circular form in double strands (II) was stable enough to its action, and was not sensitive to Zn²⁺ ions as inhibitor.

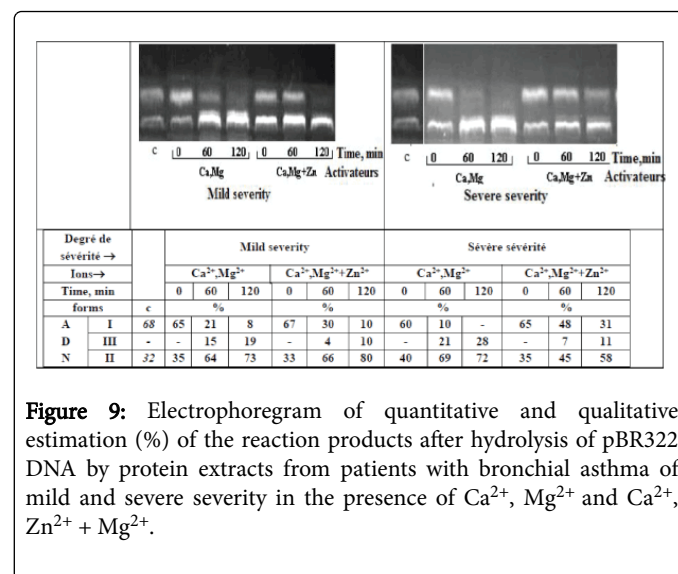


Figure 9: Electrophoregram of quantitative and qualitative estimation (%) of the reaction products after hydrolysis of pBR322 DNA by protein extracts from patients with bronchial asthma of mild and severe severity in the presence of Ca²⁺, Mg²⁺ and Ca²⁺, Zn²⁺ + Mg²⁺.

Discussion

The functionality and duration of bronchial asthma allow suggesting that one of the reasons for the chronicity of the disease is slowdown of the elimination of lymphocytes of the lungs. An analysis of previous studies on lymphocytes of patients with bronchial asthma showed the stability of these cells in the process of programmed death, which corollary is the slowdown of DNA fragmentation [30-32]. It is not excluded that this is due to the action of nucleases contained in peripheral blood lymphocytes of patients with asthma. Currently, there is convincing evidence of the existence of deoxyribonuclease involved in the apoptotic process [33]. According to some authors [34] there are two types of DNases involved in PCD 1 during the morphogenetic process in animals: DNase-dependent on metal ions (Mn²⁺ and Ca²⁺, Mg²⁺) and DNases-independent on metal ions. For other authors,

DNases involved in apoptosis may be classified into three groups: the Ca^{2+} / Mg^{2+} endonucleases, the Mg^{2+} endonuclease, and the cation-independent endonucleases [33]. Recombinant human DNase I has been developed clinically for treatment of pulmonary disease in patients with cystic fibrosis [35]. DNase I is also under consideration for a variety of other diseases, including pediatric lung diseases other than cystic fibrosis [36], systemic lupus erythematosus [37,38] and cancer [39]. According to Nevinsky et al. [40] the deoxyribonucleases are very important in the process of metabolism of nucleic acids and for maintaining the physiological concentration of DNA in the living organism [40-42]. Several studies have shown that DNase as well as the antibodies hydrolyzing DNA can be used successfully in the diagnosis of various diseases. We observed the presence of multiple DNase in lymphocytes. Khodarev et al. have shown that DNase Ca^{2+} , Mg^{2+} dependent on nucleus of lymphocytes of normal persons cleave plasmids specifically at defined locations [43]. Our data showed that the granular extracts of lymphocyte have an enzymatic activity in the presence of Ca^{2+} ions, Mg^{2+} , which is consistent with the results of Pio et al. where the granular extract hydrolyzes pBR 322 DNA with an accumulation of the circular shape with time [13]. The zinc influences this hydrolysis reaction of granular proteins of lymphocytes and its role may vary from induction to inhibition, according to the severity degree of asthma. Moreover, Pio et al. showed the existence in monocytes of nuclease similar to that found in the lymphocytes from asthmatics, and which is inactivated by Zn^{2+} ions but whose activity increases with decreasing pH of the medium [13]. This DNase occurred in the fragmentation of DNA during the process of programmed death of type 1, and was linked to an increase of Ca^{2+} ions and chelated complex associated with intracellular Zn^{2+} ions. Thus, in our present work, the expression of enzymatic activity in the presence of zinc allows us to suggest the presence of DNase acid in granules, which activity is not necessarily associated with divalent metal ions. There are data on the possibility of the involvement of the DNase activity of T-lymphocytes not only in the DNA fragmentation induced by cytotoxic T lymphocytes (CTL) to target cells, but also in the process of programmed death of type 1 of these cytotoxic cells (CTL). This idea coincides with the observations that the increase of the DNA fragmentation occurring in thymocytes, which is in relation with an increase in intracellular Ca^{2+} and Zn^{2+} ions from CTL cells. Furthermore, the granular constituents of CD8 lymphocytes CTL-granules, such as TIA-1 RNA-binding proteins have been described as part of the molecular processes involved in the cascading of programmed death of type 1 Fas-dependent. On the other hand, there are data which stipulate that the CD4 cytotoxic T-lymphocytes, inducing apoptosis in some cells (HLA-II ARC), may be directly involved in the mechanism of tissue destruction. And the answer to this abnormality occurs in autoimmune diseases. In addition, it was also found that the sub-population of CD16 and CD14 cells and DNases associated with monocytes increased in patients with some autoimmune diseases (grave disease, multiple sclerosis). It has been established that cytotoxic monocytes play an important role in tissue destruction as NO in the DNA fragmentation and cause an increase in the activity of the DNase in CD14 CD16 subpopulations potentially cytotoxic.

In summary our data show that nuclease was present in granules of lymphocytes freshly obtained from patients with bronchial asthma and may suggest that this enzyme could play some role in the programmed death of lymphocytes. This not only allows a better understanding of the morphological and biochemical changes observed during the course of apoptosis in lymphocytes of patients but also brings more to

the knowledge of the enzymatic influence in the process of type 1 programmed death. All this might be used for the diagnosis of asthma with a better accuracy on the prognosis of the severity degree.

Author's contribution

ZIA, CAV, LBM designed the study. CAV, YVS, IDR, SAA participated in the technical work and the acquisition and interpretation of data. HS, SAA, CAV evaluated the literature. CAV, ZIA, IDR, YVS carried out the experiments of this study. LBM, HS, SAA, and ZIA have given final approval of the version to be published. All authors have read and approved the final manuscript.

Acknowledgement

This work was funded by the subsidy allocated to Kazan Federal University for the project part of the state assignment in the sphere of scientific activities (No 04-03/10).

Conflict of Interest

The authors declared that they have no competing interests.

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