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Granule Associated DNase in T-Lymphocytes from Patients with Inflammatory Disease

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Abstract Recent data highlight the undeniable role of programmed cell death type I of lymphocytes in the pathogenesis of certain allergic diseases and autoimmune diseases such as Bronchial Asthma and hemorrhagic rectocolitis. But little data exist on the enzymatic activity of secretory granules associated with lymphocytes of patients suffering from these inflammatory diseases. The aim of the study was to characterize the activity of the DNase in the secretory granules of T lymphocytes isolated from peripheral blood of patients with Bronchial Asthma (n = 20) and Hemorrhagic Rectocolitis (n = 20). Thus, the secretory granules were isolated from lymphocytes by the ultracentrifugation method on percoll density gradient. The detection of the activity of the protein extracts was performed by zymography and electrophoresis method. The results reveal the presence of a protein extract with a molecular weight of 66 kDa both at the level of the granules of the lymphocytes of patients suffering from hemorrhagic rectocolitis considered as a classical autoimmune disease and in the granules of lymphocytes of patients with Bronchial Asthma. The study of physicochemical properties showed an increase in the enzymatic activity of DNase of secretory granules when 1 mM Ca^{2+} was added to the incubation medium at a pH = 7.5. On the other hand, the addition of 1 mM Zn^{2+} causes the inhibition of enzyme activity. These results suggest that the enzymatic activity of DNase detected in the granule of lymphocytes of patients with Bronchial Asthma and hemorrhagic rectocolitis occurs not only in the fragmentation of double stranded DNA but could also play a role in the apoptotic process of these same T-lymphocytes. The study of the properties of this DNase in the inflammatory diseases could enable to use this protein as a marker for determining the severity of disease.

Keywords: *DNase, secretory granules, Bronchial Asthma, Hemorrhagic Rectocolitis*

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1. Introduction

The apoptosis or programmed cell death type I is the keystone to many biological processes in multicellular organisms [1,2]. Among other things, it plays an undeniable role in the final stages of inflammation, during this period, the removal of active immune cells having completed their functions takes place. The dysfunction of this process in immunocompetent cells is one of the causes of development of allergic diseases and autoimmune diseases. For instance, one can cite Bronchial Asthma [3,4] and Hemorrhagic Rectocolitis, respectively. According to data from the literature, Bronchial Asthma is associated with the resistance of lymphocytes to apoptosis, which leads to a persistence of allergic inflammation [5,6,7]. This resistance leads to a disruption in the removal of lymphocytes from the lung tissue accompanied by a bronchial hyper-reactivity. On the other hand, the Hemorrhagic Rectocolitis is considered as an autoimmune disease caused by a disorder of the immune system that attacks its own cells. The inflammatory immune response in Hemorrhagic Rectocolitis patients is perpetuated by the maintenance of T-lymphocytes activation and the production of a cascade of inflammatory mediations. The apoptosis disorder of these lymphocytes leads to a disruption of the negative selection system. Thus, the auto-reactive lymphocytes that escaped this process can survive and attack its own cells. The tissues are infiltrated by lymphocytes and more specifically cytotoxic lymphocytes causing disruption of some biochemical processes. One of the key stages of apoptosis is the fragmentation of DNA. The DNA fragmentation is a complex biochemical process, which involves a group of nucleases with different activities and specificities for the substrate. It was detected in the secretory granules of patient lymphocytes, granular extracts of which enzymatic activity increases with the degree of severity of the disease and the balance of the regulatory activity of the immune response. The characteristics of the enzymatic activity of DNase are different from the activity of other known

endonuclease involved in DNA fragmentation (DNase I, DNase II). The DNase may be involved in both the apoptosis of its own lymphocytes and could also serve as cytotoxic factors [8]. The identification of new nucleases and the analysis of their functions in the apoptotic process and their development should lead to a comprehensive study of the links between apoptotic degradation of DNA sequences and autoimmune and allergic diseases [9]. The aim of this work was to characterize the activity of the DNase in the secretory granules of T lymphocytes of patients with Bronchial Asthma and Hemorrhagic Rectocolitis.

2. Materials and Methods

2.1. Study Purpose

The aim of the study was the activity of DNase associated with secretory granules of T lymphocytes isolated from peripheral blood of patients with Bronchial Asthma (BA, n = 20) and Hemorrhagic Rectocolitis (HR, n = 20). The choice of these diseases (BA and HR) as a model for the study is related to the fact that during the development of these diseases, an important role is played by the stability of the lymphocytes in relation to apoptosis, and moreover hitherto there is still no consensus on the importance of the autoimmune process in the pathogenesis of these diseases. The HR is referred to serious autoimmune disease with cell mediation, such as those described for the activity of DNase of granules of T-lymphocytes.

2.2. Isolation of Peripheral Blood Lymphocytes

The lymphocytes were isolated from the peripheral blood of donors relatively healthy (HD) and patients with BA and HR on a ficol-verografine density gradient from the standard method proposed by Patel et al [10] and improved by Boyum [11,12].

2.3. Isolation of Sub-populations of T- lymphocytes

The subpopulations of T- lymphocytes were obtained by negative immunomagnetic separation method with the use of a set of magnetic particles Dynabeads® Untouched ™ Human T Cells Kit (Invitrogen, USA). This method consists in isolating 95% of T lymphocytes and their viability was determined by the method of exclusion with trypan blue [13].

2.4. Obtainment Secretory Granules of Cytotoxic T-lymphocytes

The secretory granules were isolated from lymphocytes by the ultracentrifugation method proposed by Borregard [14] on Percoll density gradient (Invitrogen, USA) and then optimized by Podack et al [15] with some modifications [16]. For the preservation of the structure of the lymphocytes and for the obtaining of secretory granules, the lymphocytes were rinsed in 0.32 M sucrose solution prepared in buffer solution 0.01 M Tris-HCl at pH 7.2 and containing 0.003 M $CaCl_2$. After centrifugation at 5000 rpm/min for 20 min, we added to the pellet obtained, 1 ml buffer solution of 0.01 M Tris-HCl at pH 7.3. The cells were then suspended in the buffer solution and were all transferred in a Potter glass tube. The cells were ground in the homogenizer with the potter piston for 2 min in an ice bath, and then 3 ml buffer solution of 0.01 M Tris-HCl was added. They were incubated for 30 min in an ice bath. The substance obtained after grinding was centrifuged for 15 min at 2300 rpm / min and at 4°C. The supernatant was collected and the pellet was washed with a buffer solution of 0.01 M buffer Tris and centrifuged again for 15 min at 2300 rpm / min (K-24). The supernatant was collected again. The procedure was repeated again, after which supernatants were put together for possible extractions of granules. The granules were obtained by Percoll density gradient (ρ=1.080 g/cm). And five (5) milliliters of supernatant obtained were placed on the Percoll gradient and centrifuged at 19500 rpm/ min for 35 min with the Beckman centrifuge (L90-K, Coulter, USA). After centrifugation, 2 ml fractions 5-7 were collected and which were visible to the naked eye. For the removal of Percoll gradient these fractions were centrifuged at 45 000 rpm/ min for 2.5 hours at 4°C. A whitish supernatant was formed on the Percoll of 80-90% homogeneous supernatant containing secretory granules. This whitish supernatant was solubilized in a buffer solution for eventual use.

2.5. Extraction and Purification of Protein Extracts from Granules of T lymphocyte

In order to destroy the granule membrane the granule solution was subjected to several cycles of freezing and thawing in liquid nitrogen after storage at - 80°C overnight. For complete lysis of the membrane of the granules we added a buffer solution (1 M $NaKHPO_4$, pH 7.4, 1 mM EDTA, 3 mM NaN_3, and 2 mM PMSF) at equal volume. The mixture was incubated in a refrigerator for 30 minutes. The addition of high concentrations of phosphate led to the precipitation with particles Percoll, which were easily removed by centrifugation for 30 minutes at a speed of 20000 rpm/m at 4°C (Rotor SW 55 Ti). To precipitate lymphocyte granule proteins, the method of separation was used with ammonium sulfate. For purification of these extracts, the dialysis method was used. Dry polyethylene glycol of molecular weight 40000 g was used to enhance the concentration of protein extracts.

2.6. Disc Electrophoresis of Protein Extracts from Granules of T lymphocytes

The separation of protein extracts contained in the granules of cytotoxic lymphocytes according to their molecular weight was performed using the method of 12% disk electrophoresis under the denaturing conditions [17].

2.7. Detection of the Activity of Protein Extracts by Zymography Method

To visualize the enzymatic activity of protein extracts of granules with the simultaneous determination of the molecular weight of these extracts, zymography, a sensitive method, was used [18]. It summarize the

procedure of electrophoresis on 12% polyacrylamide gel under denaturing conditions after polymerizing native DNA in the acrylamide gel and initiated an enzyme reaction by incubation in an activation buffer solution. The gel was washed and incubated in buffer solution for 30 minutes at 37°C containing ethidium bromide at a final concentration of 1 µg/ml. The fluorescence of ethidium bromide was recorded by the gel documentation system BioRad ChemiDoc XRS + (~ 300 nm excitation, ~600 nm emission). In this context the bottom of the gel with polymerized DNA showed a black spot at the places where occurred enzymatic reactions.

2.8. Study of Cation Dependence and the pH Influence on the Activity of Protein Extracts from Granules of T lymphocytes

We studied the catalytic activity of protein extracts of granules dependent on cations by power of native DNA degradation in the presence of different concentrations of Ca^{2+} and Zn^{2+} ions. The protein extracts with a concentration of 350 µg/ml) were incubated in 50 µl of buffer solution (10 mM Tris-HCl, pH 7.5; 50 mM NaCl; 10 mM $MgCl_2$), containing 10µg of genomic DNA of the hedgehog sea and Ca^{2+} and Zn2 + ions. During the study of the influence of pH, the protein extracts of the granules were incubated for 24 hours in a buffer solution (50 mM NaCl; 10 mM $MgCl_2$) at pH = 5.0, pH 7 and pH 8.0. After incubation for 24h, we caused the migration of 20 µl of these aliquots on an agarose gel containing 1 µg/ml 1% ethidium Bromide for 3 hours with a voltage of 5V/cm.

2.9. Statistic Analysis

Statistic analysis was performed using the software package STATISTICA 6.0. For quantitative and qualitative analysis of the activity of granuleextracts, the gels were scanned and the results were processed with the "Scion image" program. We also used the excel package to analyze the data.

3. Results

3.1. Extraction of Secretors Granules of T lymphocytes

Because of the special role of T lymphocytes in the development of the immune response to some diseases such as BA and HR, we chose the negative immunomagnetic separation method to obtain a homogeneous subpopulation of T lymphocytes from a fraction of mononuclear cells from peripheral blood. Secretors granules of T lymphocytes were isolated by ultracentrifugation on a density gradient Percoll. During the design of this protocol, we were forced to adapt the procedure to the realities of our laboratory. It was necessary for us to optimize the gradient/supernatant ratio for a successful extraction of secretory granules. We considered several gradient/supernatant ratios (2: 1; 3: 1; 4: 1). The 4: 1 turns out to be the most appropriate. With such a ratio, it was observed in the Percoll gradient visible and clear layer constituted of secretory granule after centrifugation. This is presented in Figure 1. The ratios 2: 1 and 3: 1 did not show the area containing secretory granule.

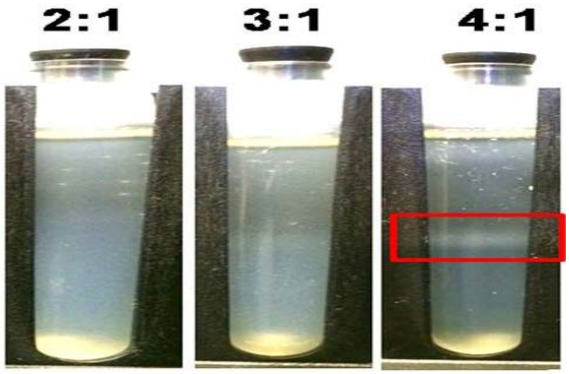

Figure 1. The conditions for an optimum choice of the ratio gradient / supernatant for the isolation of secretory granules of T lymphocytes. The clear area encircled in red shows the position of granules in the Percoll gradient for gradient / supernatant ratio 4: 1

3.2. Electrophoresis of Protein Extracts of the Secretory Granules of Lymphocytes

In order to determine the molecular weight of the protein extract contained in the secretory granules of patient lymphocytes, vertical electrophoresis was performed on a polyacrylamide gel. The electropherogram has revealed the presence of a protein extract with a molecular weight of 66 kDa both at the level of the granules of the lymphocytes of patients with HR and in granules extracted from lymphocytes of patients with BA. In short we have identified a protein extract contained in the granules with a molecular weight of 66 kDa. The electropherogram shows in the third lane, apart from one protein band with a molecular weight of 66 kDa, three additional protein bands which could be other types of protein extracts. The identification of these proteins was not included in the objective of our research work (Figure 2).

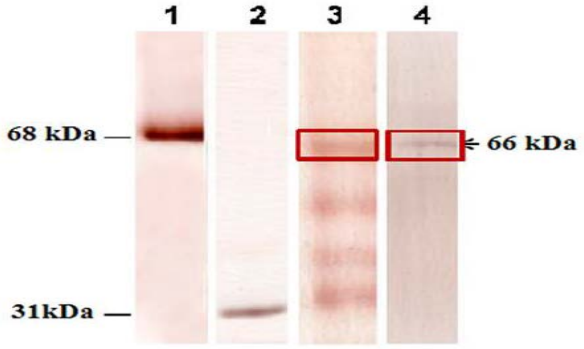

Figure 2. Electropherogramme of protein extracts of secretory granules of lymphocytes showing the molecular weight of these extracts. (Polyacrylamide gel under denaturing conditions at 12%, - revelation of protein extracts with silver nitrate - lane 1: Serum Albumin of bovine (68 kda); lane 2 DNase 1 (31kda), lane 3: protein extract from granules of lymphocytes from patients with CU (30µg); lane 4: protein extracts from granules of lymphocytes from patient with BA (30µg)

3.3. Detection and Localization of the Enzymatic Activity of Secretory Granules of T-lymphocytes

We used zymography method to verify the presence of a DNase in the lymphocyte granules with a molecular weight of 66 kDa and their enzymatic activities. This highly sensitive method was used both to separate proteins according to their molecular weight and to visualize the

enzyme activity of this enzyme protein directly into the gel that was previously polymerized with DNA (due to activation of nucleases) in the gel after electrophoresis. A commercial preparation of DNase of calf pancreas (Sigma-Aldrich, USA) served as control for the enzyme activity. The purity of this preparation was 85%, which was insufficient for zymography, as the zymogram shows in addition to the nuclease activity of DNase 1 (with a molecular weight of 31 kDa) other bands which might give a false interpretation of results. To this end, it was important for us to purify the preparation to remove impurities. For purification, we used the gel filtration chromatography method. The results of the purification of DNase are presented in the chromatogram (Figure 3). The control of the purification of protein fractions obtained from impurities after chromatography was verified by the electrophoresis method in polyacrylamide gel; the results are presented in Figure 4. The electropherogram shows many contaminants with different molecular weights at the levels of lanes B2-B4. On the other hand, at the level of lanes C4-C7, traces of impurities were observed. As a matter of fact, we determined DNase activity found in lanes B8-C3 by zymography method. The fractions of lanes C1-C2 DNase were used as controls to verify enzyme activity of the protein fractions of isolated granules from T lymphocytes of patients with BA and HR (Figure 5). Zymography was performed on 12% gel of SDS-polyacrylamide polymerized with native DNA (15µg/ml) for a period of 18 hours. The zymogram has shown an enzymatic activity of protein fractions of lymphocyte granules corresponding to proteins with a molecular weight of 66 kDa (Figure 6).

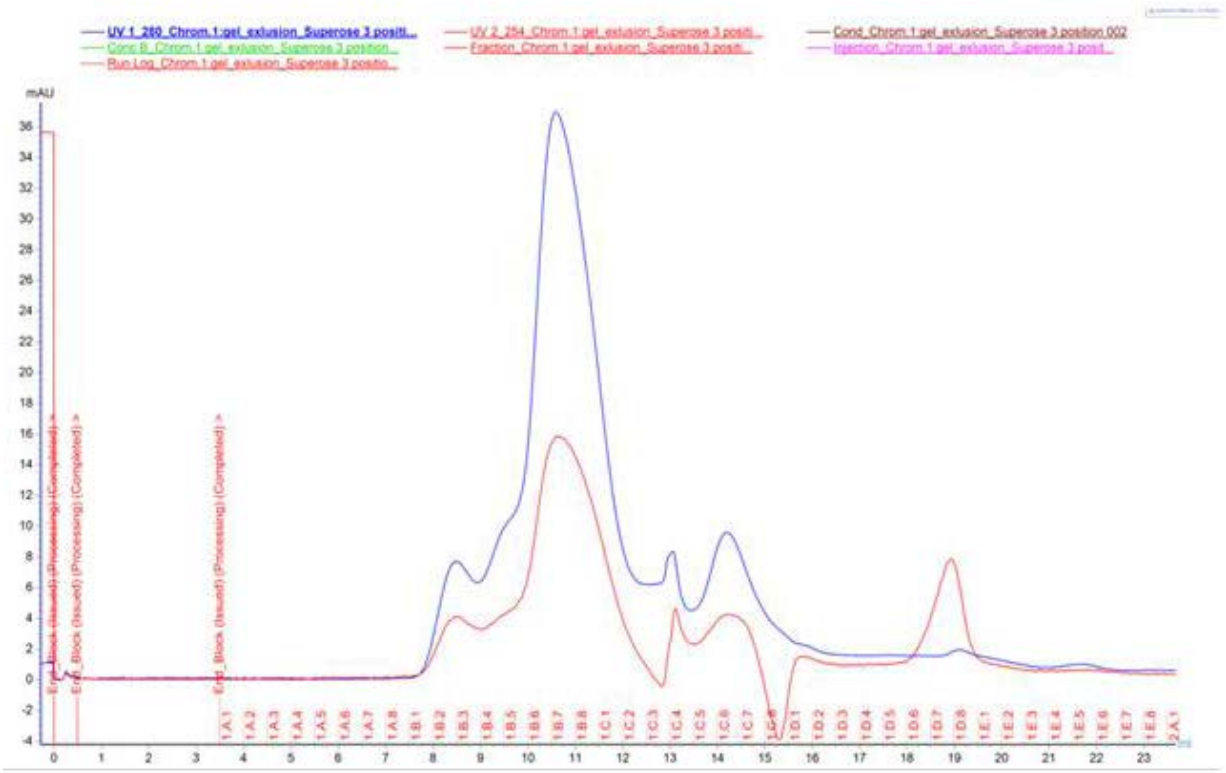

Figure 3. chromatogramme of protein after gel filtration, of a commercial preparation of DNase I

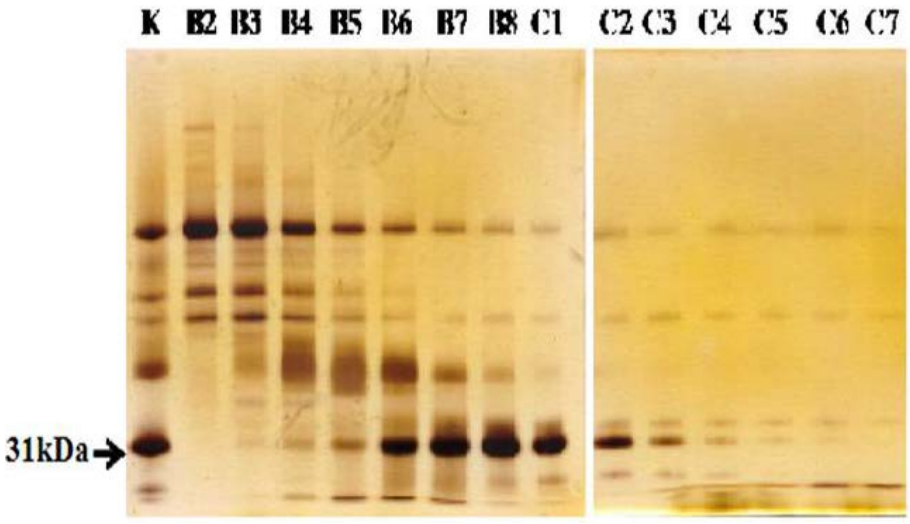

Figure 4. Electropherogramme of protein fractions after gel filtration of commercial DNase. Lane K; DNase before purification; B2-C2: DNAse after the chromatography method of gel filtration; lane B8-C3: DNase free of almost all impurities

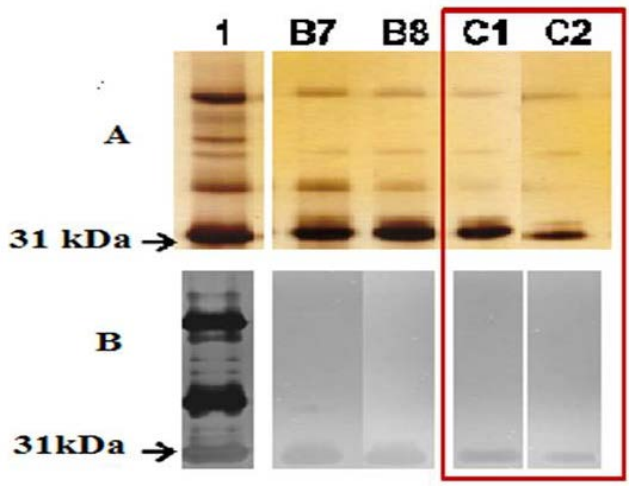

Figure 5. Electropherogramme (A) and zymogramme (B) of purified fractions of commercial DNase I. lane 1: commercial DNase before purification. Lanes B2-C2: purified DNase fraction; lanes C1-C2: DNase fraction retained for use as a control for the following experiments

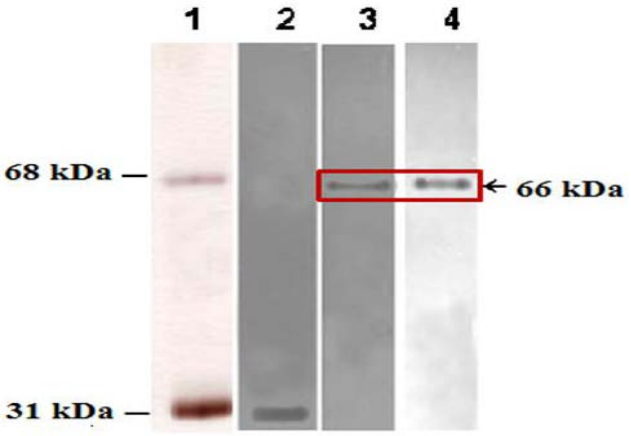

Figure 6. Zymogramme showing the enzymatic activity of protein extracts of granules from T lymphocytes. Lane 1 - markers (BSA, 68 kD; DNase I-31 kDa), revealed with silver nitrate; lane 2 - purified commercial DNase I; lane 3 - protein extracts of lymphocyte granules of patients with BA (30µg); lane 4 - protein extract of lymphocyte granules of patients suffering from CS (30 µg). The red band shows the demonstration of the enzymatic activity of DNase corresponding to a protein with a molecular weight of 66 kDa

3.4. Study of Physico-chemical Properties of Protein Extracts of the T-lymphocyte Secretory Granules

To determine the physical and chemical properties of the protein extracts secreted by granules of T lymphocytes, we examined the dependence of cations such as Ca^{2+} and Zn^{2+} and the influence of pH of the reaction medium. Therefore, we determined the influence of cations on the enzyme activity of protein extracts by studying the degree of degradation of the commercial DNA of sea urchin (10 µg) with protein extracts secreted by the granules after an incubation in a buffer solution (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) containing Ca^{2+} and Zn^{2+} ions. The incubation was performed at 37°C for 24 hours and the results are presented in Figure 7. We observed that the enzymatic activity of protein extracts of T lymphocytes of patients suffering from BA and HR increases when it is added to the incubation medium 1 mM Ca^{2+} and the activity decreases when 10 mM Ca^{2+} was added to the medium. After addition of 1 nM Zn^{2+} the enzymatic activity of the protein extracts of T lymphocytes decreased in both cases. The influence of the pH of the medium on the enzymatic activity of the protein extracts of lymphocyte granules was determined after incubation of native DNA of the sea urchin (10 µg with protein extracts of granules in a buffer solution (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) with pH of the medium slightly acidic (pH = 5.0), neutral (pH = 7.0) and slightly basic (pH = 8.0). the incubation was carried out at 37 degrees Celsius for 24 hours. There was an effect of pH on the activity of protein extracts of granules isolated from lymphocytes of patients with BA and HR. The enzymatic activity became maximal after a slightly acidic medium around 5.0.

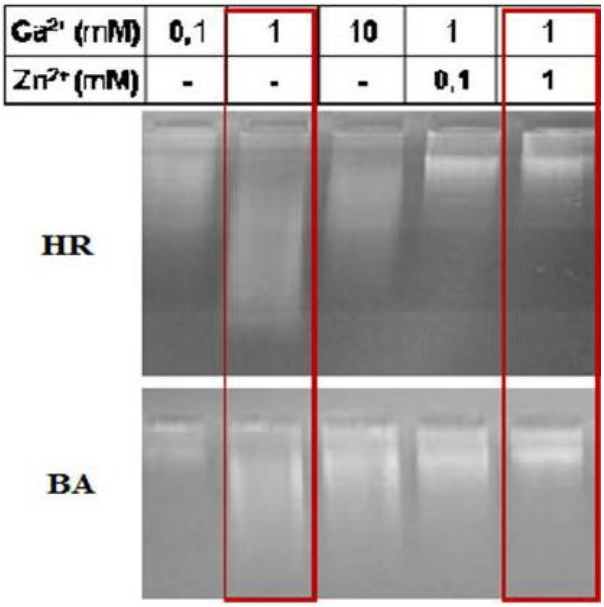

Figure 7. Electropherogram showing the influence of the cations on the protein extracts lymphocyte granules of patients with BA and patients suffering from CR. The DNA was incubated in a buffer solution with different concentration of Ca^{2+} and Zn^{2+}. The lanes marked in red shows the activating effect of 1 mM Ca^{2+} and inhibitor effect of 1 mM Zn^{2+}

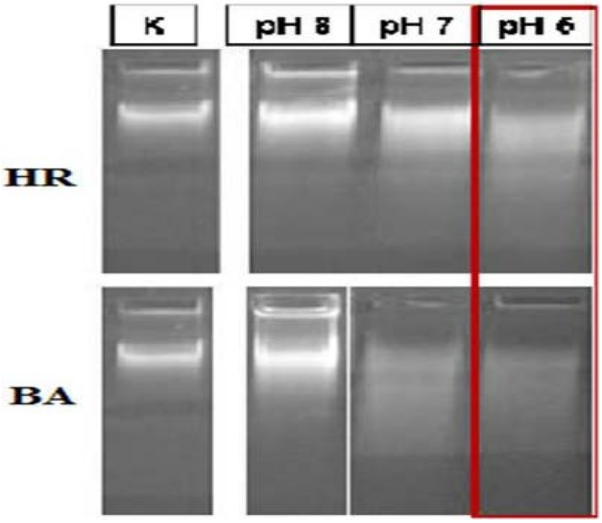

Figure 8. Electropherogramme showing influence of pH of the medium after incubation of the DNA of the sea urchin with protein extracts of lymphocyte granules of patients with BA and CR in a buffer solution (10 mM Tris-HCl; 10 mM Mg2: 50 mM + 1 mM $CaCl_2$)

4. Discussion

The apoptosis and autoimmunity are potentially important factors playing a curable role in the pathogenesis of some serious diseases [19,20]. Apart from these factors, one can also think of the lymphocytes that constitute the keystone and which are involved in almost all immune reactions and autoimmune as well as allergic reactions [21]. The inflammatory reactions in asthmatics are characterized by delayed-type hypersensitivity reactions [22]. Therefore, there is a selective accumulation of T lymphocytes in the respiratory tract. The accumulation of these lymphocytes in the respiratory tract is due to the malfunction of the apoptotic process of these cells. Some authors argue that apoptosis of eosinophils and T lymphocytes with cellular mediation is a key pathogenic event leading to the loss of epithelial cells in asthma patients. The key stage of apoptosis of these cells is the stage of fragmentation of nuclear DNA. And DNA degradation is due to the activity of various enzymes. A failure of the activity of these enzymes may cause malfunction of the apoptotic process and in particular its inhibition. It has been described the enzymatic activity of DNase associated with cytoplasmic granules of activated T lymphocytes after incubation of mononuclear cells from peripheral blood of relatively healthy donors in the presence of immunomodulatory peptides. The induction of their activity in human cytotoxic lymphocytes, particularly T-lymphocytes during the development of autoimmune diseases has led us to study the possibility of eventual presence of granular DNase found in the lymphocytes of peripheral blood from patients with asthma and HR. Some common elements in the development of asthma and specific autoimmune diseases of organs (for instance, infiltration of T- lymphocytes in the outbreak of the disease) led us to study the activity of the nuclease of secretory granules of peripheral blood lymphocytes of patients with BA and HR. The results of our research allowed us to detect the presence of DNase with enzymatic activity in the secretory granules of T-lymphocytes of patients with BA and HR. The method of zymography a very sensitive method was used to visualize the activity of this DNase in a polymerized polyacrylamide gel with double-stranded DNA. The protein identified in the granules had a molecular weight of 66 kDa if we confine ourselves to the nuclease activity of the enzymatic protein. From the study of physicochemical properties, an increase in the enzymatic activity of DNase of secretory granules when added 1 mM Ca^{2+} to the incubation medium and pH = 7.5 was observed. On the other hand, the addition of 1 mM Zn^{2+} causes the inhibition of enzyme activity. The activity of the protein fractions contained in the lymphocyte granules of BA patients and HR increases after addition of Ca^{2+} ions to the incubation medium with an acidic pH. Thus, it was observed in the secretory granules of T lymphocytes the presence of DNase corresponding to Ca^{2+} ion-dependent DNase with a molecular weight of 66 kDa, of which the activity is more important at a slightly acidic pH. This DNase associated with granules corresponds to a protein band detected by Lopez Moratalla [8]. But the role played by this DNase is not yet elucidated clearly, and one might assume that this could be another cytolytic factor. This hypothesis is consistent with the idea that the expression of enzymes in the secretory granules could be associated with potentially cytolytic T lymphocytes, and the mechanism of cytolytic T lymphocytes is linked to an active transfer of fragmented DNA by cytotoxic factors in the target cells. In this respect, one might assume that DNase detected in the granules is not only involved in the fragmentation of double-stranded DNA of the target cells but also in the apoptotic process of the same T lymphocytes. This hypothesis coincides with the fact that, in the thymocytes the increase in the concentration of intracellular Ca^{2+} and the connection with the Zn^{2+} ions [23] cause an increase in the DNA fragmentation due to the acidification of the medium. On the other hand, it has been shown that cytotoxic T lymphocytes could be involved in damage to target tissues. For example, the activity of DNase associated with secretory granules of T lymphocytes [8] which increase in patients with primary biliary cirrhosis of the liver but whose activity is lower in patients with Basedow disease which correlates with the highest immunoregulatory disorders. Thus, the detected DNase could play an exceptionable role not only in the autoimmune diseases but also in the diseases such as bronchial asthma. Many researchers have studied the role of autoimmunity in the pathogenesis of bronchial asthma by the functioning of T lymphocytes [19,24]. It was thus discovered molecules that can initiate, carry out the apoptosis of lymphoid cells in the course of certain diseases, but it should be noted that stimulants leading to their action and mechanisms used in these cases are limited. However, since the induction or inhibition of apoptosis is targeted as an ideal way of treating certain pathologic diseases, the development and research in this area should be considered a high priority. In the same vein, the study of apoptosis of lymphoid cells in the course of bronchial asthma is interesting and relevant. Henceforth, the discovery of a DNase with a molecular weight of 66 kDa in the granules of T lymphocytes from patients with BA and HR coincides with these characteristics with that previously studied by PIO et *al* in patients with severe autoimmune disease. The presence of such DNase molecules in asthmatics indicates the role of the autoimmune process in the pathogenesis of BA. Further study of the functions of this molecule would help to establish a link between the apoptotic degradation of DNA and disease, and the study of its activity according to the form and degree of disease severity would permit the use this DNAse as marker of immune system deregulation. This could help us justify the paradigm of bronchial asthma is an autoimmune disease.

5. Conclusion

In summary our data show that DNase was present in granules of lymphocytes freshly obtained from patients with BA and HR and may suggest that this DNAse could play some role in the apoptosis of T-lymphocytes. All this might be used for the diagnosis of inflammatory disease with a better accuracy on the prognosis of the severity degree.

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Competing Interests

The authors declare that they have no competing interests.

All authors have read and approved the final manuscript.

Ethics Approval

Experimental research has been performed with the approval of an appropriate ethics committee. The work was done jointly with medical doctor who received approval from the Local Ethics Committee of the Medical University of Kazan for the conduct of biomedical research. The work was performed in accordance with the rules of the Ethics Committee in the laboratory of Clinical Immunology and Allergy of RKB. All patients were informed and they gave their consent for experiment.

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