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The Particularities of Protein fraction in the Apoptosis of Lymphocytes of Patients with Asthma

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Abstract: The emergence of various diseases specifically severe diseases such as bronchial asthma is associated with apoptosis of lymphocytes. One of the major biochemical features of apoptosis is chromosome DNA fragmentation implemented by apoptotic nucleases. The inactivation of these apoptotic nucleases produces undigested DNA and is linked to a number of autoimmune disorders. Instead of this we have studied the enzymatic activities of the cytoplasmic and nucleic proteins of lymphocytes from healthy donors and patients with bronchial asthma. The study of enzymatic activities of the nuclease of lymphocytes was assessed by flow cytometry, spectrofluorimetry and electrophoresis method in agarose gel. In the peripheral blood cells of healthy donors undergoing apoptosis, we found a DNAse activated by Ca^{2+} and Mg^{2+} ions. Lymphocytes of patients with bronchial asthma contain DNAses, the activity of which depends on the seriousness of the desease. In patients cells, the activity of the Mn^{2+}-dependent DNAse increases, whereas the activity of the Ca^{2+}, Mg^{2+}-dependent DNase decreases. Taking into consideration the role of the Ca^{2+}, Mg^{2+}-dependent DNAse in apoptosis, we can propose that there is a link between the reduction of the rate of apoptosis of lymphocytes in patients with bronchial asthma and the dysfunction of the induction of "apoptotical" Ca^{2+}, Mg^{2+}-dependent nuclease. According to the results obtained, we can assume that why apoptosis of lymphocytes resist in patients with bronchial asthma is a reduction in concentration of endocellular calcium and an increase of manganese ions content, which results in the triggering of activation mechanism for Mn^{2+}-dependent endonuclease activity. This leads to the change of DNA fragmentation nature in lymphocytes and as a consequence, to disorders in process of apoptotic bodies' formation, thus, hindering apoptosis of lymphocytes in patients with bronchial asthma.

Key words: Apoptosis, DNA, protein fraction

INTRODUCTION

Apoptosis is a highly conserved and integrated response in normal physiological processes (Nagata, 1997; Jacobson *et al*., 1997; Barber, 2001) of the cell with the morphological and biochemical characteristics and which plays a central role in the development of all living organisms (Kerr *et al*., 1972; Wyllie, 1980; Khorshid and Moshref, 2006). One of the major biochemical features of apoptosis is chromosome DNA fragmentation (Rottem and Shoenfeld, 2003; Urbonien *et al*., 2005) implemented by apoptotic nucleases.

Deoxyribonucleases have a significant importance in the process of the exchange of nucleic acids (Gumulak-Smith *et al*., 2001; Schmidt *et al*., 2007) and for the maintaining of the physiological concentration of DNA in the human body (Baranovski *et al*., 2004). Many studies have shown that DNAse, such as antibodies hydrolyzing DNA can be successfully used in the

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diagnosis of various pathological diseases. Currently, there is a convincing evidence of the existence of deoxyribonuclease involved in the apoptotic process. According to some authors (Boulares and Ren, 2004; Shastina, 2004) there are two types of DNAses involved in apoptosis during the morphogenetic process in animals: DNAse-dependent on metal ions (Mn^{2+} and Ca^{2+}, Mg^{2+}) and DNAes-independent on metal ions. For the others 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. The investigation of the apoptosis processes of lymphocytes in patients with bronchial asthma confirms a relative resistance of such cells to apoptosis which shows itself in deceleration of DNA fragmentation in such cells during in vitro incubation (Green and Scott, 1994; Melis *et al*., 2002). Considering this information the attention paid to the role of nucleases in cell apoptosis is quite self-explanatory. Polish scientists (Szopa and Adamiec, 1993) have hypothesized that the most important stage of apoptosis is nucleases activation. The research (Deng and Podack, 1995) shows that physicochemical characteristics of two nucleases- nuc-58 and nuc-40 enable us to call them DNases associated with apoptosis in cytolytic T-lymphocytes. It was later proved that activity of nucleases (Enari *et al*., 1998) in cells can depend on two protein molecules-not proven apoptotic nuclease (mole weight-40000) and acidic protein (mole weight-30000) which stabilizes and inactivates the nuclease. It was reported in the research (Yakovlev *et al*., 2000) that ethere is a human DNase differing from other assumed apoptotic nucleases by its nuclear localization identification. It was suggested that the DNase acting as apoptotic could be one of the following: RpS3, DNA repairing endonuclease (Jang *et al*., 2004), gamma DNase taken from thymocytes nuclei at the apoptosis phase induced by radioactive exposure, which was later found in apoptosis of T-lymphocytes in Kikuchi's disease (Higami *et al*., 2003). Taking the above mentioned into consideration we have studied the nuclease activity of lymphocytes-basic effector cells of immune system of patients with bronchial asthma.

MATERIALS AND METHODS

Patients and blood sampling: The study was carried out on the lymphocytes isolated from peripheral blood of relatively healthy (20) and asthmatic individuals (20). All the donors were non-smokers and did not receive corticosteroids within 2 weeks before the study and they were selected after consent. These patients were hospitalized in the department of pneumology. The work was performed in accordance with the rules of the Ethics Committee in the laboratory of Clinical Immunology and Allergy of RKB.

Lymphocyte isolation: Lymphocytes were isolated by centrifugation on equilibrium preformed Percoll gradient (1.105, 1.095, 1.090, 1.085, 1.077 and 1.070). The purification of the obtained fraction of the lymphocyte was 85-98%.

Flow cytometric analysis: Evaluation of lymphocyte apoptosis was carried out by flow cytofluorometry using a Facscàn (Becton Dickinson) apparatus capable of measuring the percentage of apoptotic nuclei after coloring it with propidium iodate (Nicoletti *et al*., 1991; Ismail-Zade *et al*., 2005). Nuclei apoptosis is determined via measuring the peak of hypodiploid DNA easily differentiated from a narrow peak of cells with normal (diploid) DNA. To investigate apoptosis the lymphocytes were moved into RPMI-1640 medium containing 10% of fetal calf serum, L-glutamine (Soroka *et al*., 2007) and antibiotics, incubated for 24 h in 5% CO_2 atmosphere at 37°C (Boychuk *et al*., 2003; Doering *et al*., 2004; Alode *et al*., 2011). Dexamethasone added into the culture medium to reach 5 μm concentrations was used as apoptosis inductors.

Extraction of protein fraction: To get protein fractions the method of fractioning of lymphocytes chromatin proteins was implemented. This method is based on consecutive dissociation of cytoplasmic and nuclear proteins in NaCl solutions of increasing ionic strength. As a result during the step-by-step increase of ionic strength of a solution only certain protein fractions are dissociated. For the dissociation, 0.01, 0.15, 0.4 M NaCl solutions in 0.01 M Tris buffer of pH 7.2 were used.

Determination of DNA fragmentation: To amplify pBR322 DNA we have used *Eschericiha coli*/Hb 101-pBR322 bacterial culture. Bacteria collection, their lysis and separation were done according to the method Maniatis *et al*. (1984). pBR322 DNA was purified from RNA by means of gel-chromatography on Sepharose CL-4B spin column. The enzymatic activity of the protein fractions under analysis was determined by increase in DNA hydrolysis products. This activity was expressed in spectrofluorimetric units of increase in acid-soluble products while hydrolyzing native DNA or denatured DNA at the wavelength of 260 nm within 60 min of incubation at 37°C per 1 mg of protein (specific activity) or per 1 mL of enzyme solution (total activity). The

specificity of DNAses ratio to the secondary DNA structure was investigated by electrophoresis in 0.7% agarose gel using supercoiled pBR322 DNA (form I) and has registered the transfer of form I through form II (open covalently closed circle) to form III (linear DNA). Incubation sample contained 100 μL of pBR322 DNA (20-50 μg mL^{-1})/60 μL of 0.2M Tris-buffer (pH 7.2-7.3) /20 μL of 0.025 $MnCl_2$ (50 μmol) and $CaCl_2$ (1 μmol). The concentration of ferment protein in fractions of healthy and sick donors under analysis was equalized and amounted to 0.45-1.0 μg mL^{-1}. The reaction was terminated by adding solution of 10% Na-dodecylsulfate /0.1% bromphenol blue /50% glycerine. DNA-probe containing a mixture of forms I, II and III served as transfer check of DNA forms. Electrophoresis was carried out in a horizontal direction on 10x10x3 mm plates at current intensity of 4 mA/cm within 1.0-1.5 h at 20°C. Some protein bands were discovered in the ultrachemiscope after gels coloring in ethidium bromide solution (2 μg mL^{-1}) within 40 min. The quantitative evaluation of DNases activity change (DNA hydrolysis speed) of patients with bronchial asthma depending on the stage of the disease and the donor's health was made by a fluorometric method (Winter, 1969), with determination of Ò1/2-time (in seconds) over which 50% of DNA molecules (form I) acquire single-strand breaks. To estimate the results of electrophoretic analysis for the reaction products received in vitro we have used Image Master Program (Pharmacia Biotech). The scanning of electrophoretograms was made with Sharp JX-330. As a result of the electrophoretograms scanning we have made a graphic (with peaks) representation of DNA transfer forms and have assessed their area in percentage terms.

Statistic: All these analyses were performed using Excel program and statistics software 5.0. To estimate the validity of the test data obtained we have used two-sample t-test for various dispersions. Photocopies electrophoregrams processed using a computer scanner Sharp JX-330.

RESULTS AND DISCUSSION

Apoptotizing cells are characterized by internucleosome fragmentation of nuclear DNA, leading to *in vivo* formation of apoptotic bodies. We have investigated optic characteristics of the lymphocytes separated from patients' peripheral blood (indices of forward and lateral scattering - FSC and SCC) which were different from the cells characteristics with regard to healthy donors. The research results have revealed that the incubation of peripheral blood lymphocytes in patients with bronchial asthma and healthy donors in vitro leads to cell death in course of time. Part of cells dies as a result of apoptosis. It was noted that the number of cells (in %) with hypodiploid DNA is after 72 h of incubation among lymphocytes in healthy donors higher than that among lymphocytes in patients with bronchial asthma (Fig. 1). In case of induced apoptosis in patients' lymphocytes the number of cells with hypodiploid zone increased slightly, as compared to lymphocytes of healthy donors, which have proved stability of lymphocytes in patients with bronchial asthma to spontaneous apoptosis (Fig. 2). The lymphocytes fraction obtained was used to study nuclease activity of proteins. Fractionation of human lymphocytes proteins is marked not only by certain difficulties due to small amount of cells obtained but also and by a problem of preserving nuclei integrity during cytoplasmic and nuclear proteins extraction. We have used fractionation method of chromatin proteins based on consecutive proteins dissociation in NaCl solutions of increasing ionic strength and have used 0.01, 0.15, 0.4 M NaCl solutions in 0.01M Tris buffer of pH 7.2 for that purpose. Thus, there were received three protein fractions of lymphocytes: fraction 0.01 (cytoplasm proteins), fraction 0.15 and fraction 0.4. In the course of determining DNases activity in them it was revealed that after Ca^{2+} and Mg^{2+} ions were adding to patients' lymphocytes homogenate (Fig. 3), DNases activity was not found. While adding Mn^{2+} ions the homogenate proteins hydrolysed slightly high-polymeric native DNA (Fig. 3), however hydrolyzed extensively enough denatured DNA. Specific activity of Mn^{2+}-dependent homogenate DNase activity made approximately 4.0 U per mg of protein. In separate protein fractions DNase activity was found, especially in fraction 0.4 it made approximately 50 U per mg of protein. Thus it should be noted that patients' lymphocytes are characterized by a more intensive Mn^{2+}-dependent DNase activity. To investigate the ratio of DNase activity on the secondary structure of DNA, we have used plasmid DNA pBR322 that was 90% represented by supercoiled DNA (form I) and activators-ions Ca^{2+}, Mg^{2+} and Mn^{2+}. The results have revealed that in presence of Na^{2+} and Mg^{2+} ions all fractions of patients' lymphocytes proteins showed DNase activity (Fig. 4). However products reaction was registered only 15-30 min after the beginning of the reaction. It should be noted that if in the course of adding to supercoiled DNA (I form) of cytoplasm proteins (form 0.01) (Fig. 4) in 30 min apart from circular open DNA (form II) there appear molecules of linear DNA (form III). In fraction 0.4 there was not found DNase activity transferring DNA of form II to linear DNA (form III) (Fig. 4). In all cases DNase of healthy donors' lymphocytes in presence of $Cà^{2+}$ and Mg^{2+} ions

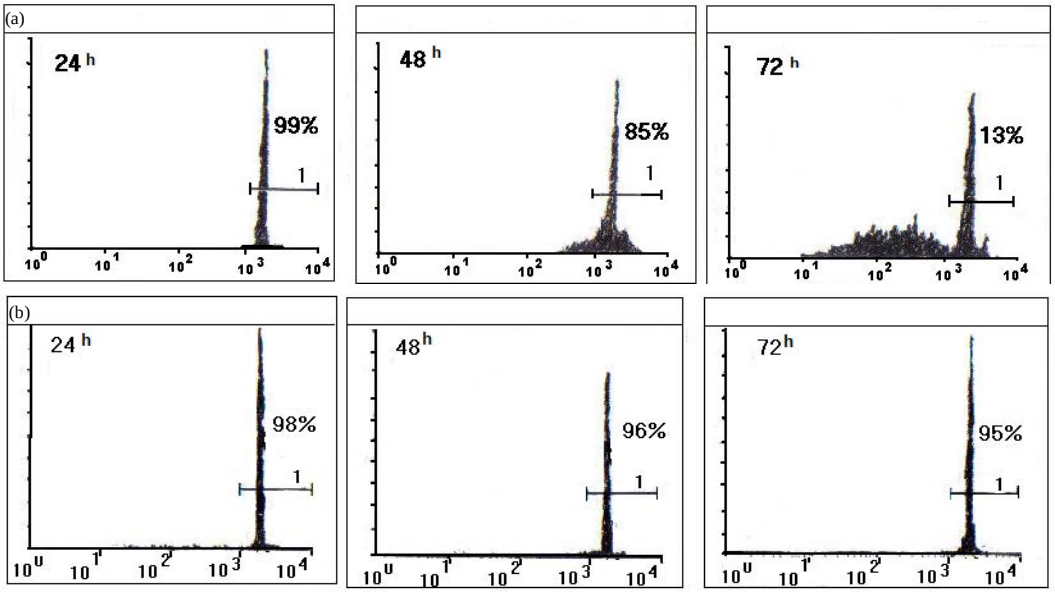


Fig. 1(a-b): Intensity of spontaneous apoptosis of peripheral blood lymphocytes in (a) Healthy donors and in (b) Asthmatic donor. Estimation of number of cells (in %) with hypodiploid DNA (number of hypodiploid DNA is estimated according to the peak width on histogram)

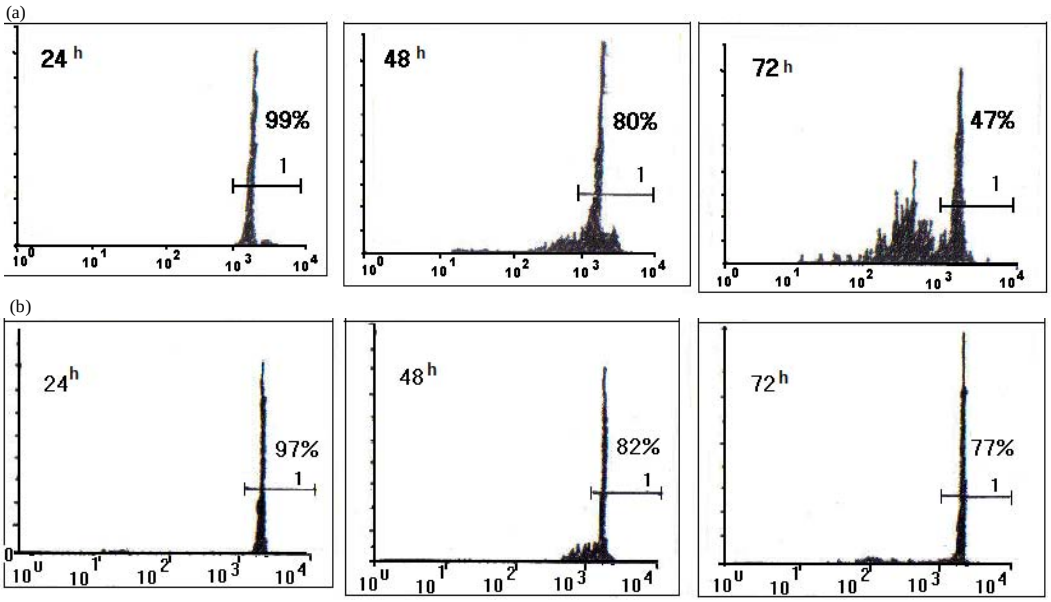
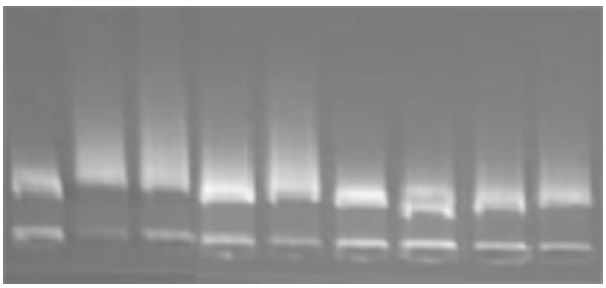


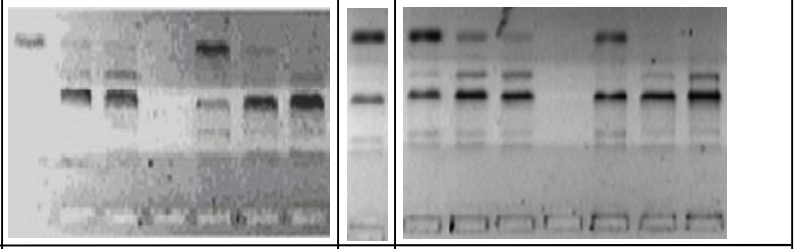
Fig. 2(a-b): Intensity of induced apoptosis of peripheral blood lymphocytes in (a) Healthy donors and in (b) Asthmatic donor. Estimation of number of cells (in %) with hypodiploid DNA (number of hypodiploid DNA is estimated according to the peak width on histogram). in asthmatics

hydrolysed the substrate more intensively than DNase of lymphocytes in patients with bronchial asthma (Fig. 4). We could observe a different picture during DNA incubation with protein fractions of lymphocytes in presence of Mn^{2+} ions. Products reaction was revealed now in 0.5-2.0 min, where DNases of fraction 0.01 (Fig. 5) and 0.15 (Fig. 6) hydrolyzed supercoiled DNA up to linear molecules and DNases of fraction 0.4 (Fig. 7) hydrolyzed



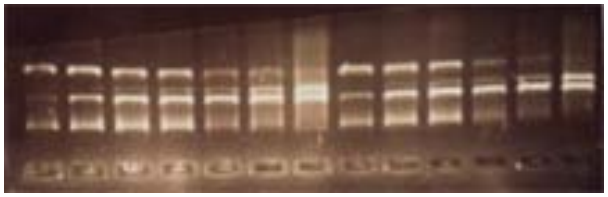
	c	2	3	4	5	6	7	8	9
Ca^{2+}; Mg^{2} (1 mM; 50mM)			+		+		+		+
Mn^{2+}, (25 mM)		+		+		+		+	
Fraction 0.01		+	+			+	+		
Fraction 0.4				+	+			+	+
		Health donor				Asthma donor			

Fig. 3: Electropherogram showing the hydrolysis products of high-polymeric native DNA by the protein fractions 0.01 and 0.4 of lymphocytes of health donor and asthmatic patients in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+}, - +; presence, -c- control



Time	0	30	60		0	30	60	c	0	30	60		0	30	60
Donor	Health donor				Asthma donor				Health donor				Asthma donor		
	Protein fraction (0.01)								Protein fraction (0.4)						

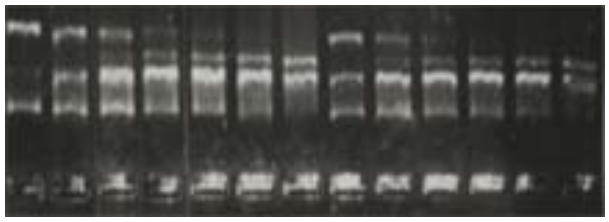
Fig. 4: Electrophoregram of pBR 322 DNA after hydrolysis of protein fractions (0.01) and protein fraction 0.4 lymphocytes of Health donor and patients of asthma in the presence of Ca^{2+}, Mg^{2+}



		Health donor							Asthma donor					
Time (min)	C	0	2.5	5	10	20	60	0	2.5	5	10	20	60
Forms	%	%	%	%	%	%	%	%	%	%	%	%	%
D	I	78	63	56	54	27	20	12	74	54	40	32	11	2
N	II	22	37	40	38	57	39	39	26	40	50	54	67	51
A	III			4	8	16	41	49		6	10	14	22	47

Fig. 5: Hydrolysis products of DNA pBR322 with lymphocytes cytoplasm proteins (fraction 0.01) in presence of Mn^{2+} ions and the quantitative evaluation of products reactions from hydrolysis of pBR322 DNA though fraction 0.01 proteins. C-substrate before the reaction starts

supercoiled DNA pBR322 only up to 'open' circle. This allowed us to make the suggestion that DNase of fraction 0.4 of lymphocytes prefers denatured DNA and is characterized by endonuclease type. Small amount of



		Health donor							Asthma donor					
Time (min)	C	0	2.5	5	10	20	60	0	2.5	5	10	20	60
Forms	%	%	%	%	%	%	%	%	%	%	%	%	%
D	I	77	64	22	12	5	3	2	62	35	13	11	5	2
N	II	20	34	73	80	85	82	70	38	60	59	57	59	39
A	III			5	8	10	15	28		5	28	32	36	39

Fig. 6: Electrophoregram of pBR322 DNA after hydrolysis though proteins of fraction 0.15 of lymphocytes in presence of Mn^{2+} ions and the quantitative evaluation of products reaction from hydrolysis of pBR322 DNA with fraction 0.15 proteins. C-substrate before the reaction starts

		Health donor							Asthma donor					
Time (min)	C	0	2.5	5	10	20	60	0	2.5	5	10	20	60
Forms	%	%	%	%	%	%	%	%	%	%	%	%	%
D	I	77	64						63	1				
N	II	23	36	100	100	100	100	100	37	99	100	100	100	100
A	III													

Fig. 7: Electrophoregram of pBR322 DNA after hydrolysis though proteins of fraction 0.4 of lymphocytes in presence of Mn^{2+} ions and the quantitative evaluation of products reaction from hydrolysis of pBR322 DNA with fraction 0.4 proteins. C-substrate before the reaction starts

linear molecules appeared only 60 min after the reaction began (Fig. 7). The continuation of incubation up to 120 min did not result in a complete transition of circular DNA to a linear one. Such resistance of relaxed circular DNA to the action of DNase of fraction 0.4 of lymphocytes cannot be explained by a protein underdose (0.45 μg mL^{-1}), because an addition of ten-fold amount of protein-clinging enzyme leads to no significant increase of linear DNA amount (form III). That is why the resistance of DNA form II to the discovered enzyme can be explained by the fact that DNase is specific to a single-stranded DNA element and during the transition of DNA forms I to form II as a result of singular break, when an instantaneous hydrogen bonds recovery occurs in denatured elements, such DNA acquires resistance to such enzyme. Thus, DNase of fraction 0.4 of lymphocytes resembles Mn^{2+}-dependent endoDNase of a rat liver described earlier by Kazan University (Belyaev *et al*., 1970; Vinter *et al*., 1993). Quantitative evaluation of products reaction (Fig. 5-7) showed that DNases of cytoplasm (fraction 0.01) and chromatin (fraction 0.4) in lymphocytes of patients with bronchial asthma and healthy donors differ slightly in terms of activity.

Accordingly, the number of molecules of ethidium bromide bound by nucleotide to linear DNA is always larger than the number of molecules of ethidium bromide bound to close circular DNA of equivalent molecular mass. Thus, a single break in circular DNA as a result of DNases action leads to the appearance of loose ends of DNA, which is accompanied by an increase of number of ethidium bromide molecules bound to DNA and, respectively, fluorescence intensity. Apart from the determination of endonuclease activity this method allows us to estimate the purity of endonuclease preparations. If

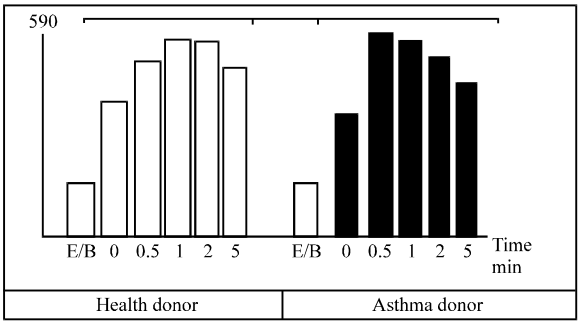


Fig. 8: Kinetics in hydrolysis of supercoiled DNA pBR322 though lymphocytes of chromatin proteins (fraction 0.15) depending on incubation time; e/b-ethidium bromide

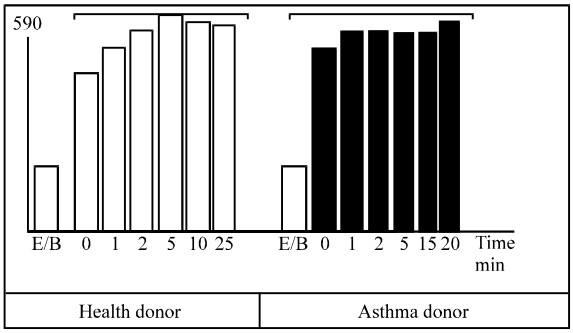


Fig. 9: Kinetics in hydrolysis of supercoiled DNA pBR322 though lymphocytes of chromatin proteins (fraction 0.4) depending on incubation time; e/b-ethidium bromide

the endonuclease preparation comprises exonuclease activity, after the endonuclease action linear molecules of DNA become accessible to exonuclease action. Hydrolysis of DNA by exonucleases leads to formation of mono- and oligonucleotide, as a result of which we can observe the reduction in sizes of nucleic acids fragments and the decrease of fluorescence intensity.

Using fluorescence method to compare qualities of DNases of fraction 0.15 and fraction 0.4 from lymphocytes of patients with bronchial asthma and healthy donors, we have proved the presence of differences in qualities of theses enzymes during substrate hydrolysis-supercoiled DNA. Figure 8 shows that as a result of supercoiled DNA hydrolysis of DNase fraction 0.15 first occurs fluorescence enhancement, which attests the formation of relaxed circular DNA and then happens significant decrease of fluorescence intensity, in other words DNase and DNases of fraction 0.15 are capable of hydrolyzing DNA forms I not only to relaxed closed circle (form II) and linear DNA (form III), however, to shorter fragments of molecules comprising smaller amount of ethidium bromide molecules than linear DNA (form III). The results given in Fig. 9 confirm those from Fig. 7 that DNase of fraction 0.4 in patients' lymphocytes is endonuclease which produces single-stranded breaks, mainly in denatured segment of supercoiled circular molecules of DNA (form I). "Open" circular molecules of DNA (form II) obtained are resistant to action of such enzyme. As it is clearly shown on the from nature of diagram (Fig. 9), DNases of lymphocytes from patients and healthy donors are distinguished by hydrolysis speed of supercoiled DNA and specificity to DNA secondary structure, which is evidenced by dynamics in rising intensity of fluorescence and the pattern of single-stranded breaks generation in DNA by proteins of fraction 0.4 from healthy donors lymphocytes is similar to pattern of hydrolysis of supercoiled DNA by proteins of fraction 0.15. To study changes in DNase activity of fraction 0.4 (substrate hydrolysis speeds) depending on severity level of asthma there was selected 2 groups with 9 persons each. The group of patients with bronchial asthma comprised 3 persons with mild stage of disease, 3 persons with medium stage of disease severity and 3 persons with severe course of disease. Kinetics in hydrolysis of DNA pBR322 by proteins of combined fractions 0.4 (obtained by fluorometric method of determining of endonuclease activity) is showed in Fig. 10. Results showed that an average of $T_{1/2}$ of patients is equal to 53.56±36.29 sec and that of

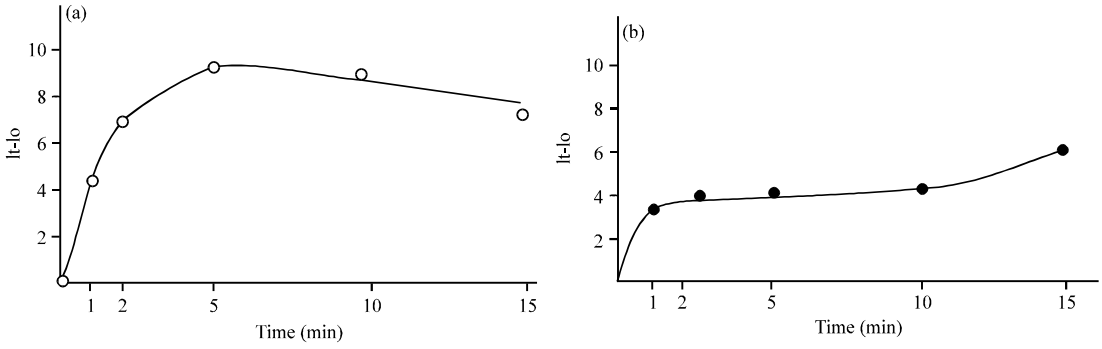


Fig. 10(a-b): Kinetics in hydrolysis of DNA pBR322 by chromatin proteins in lymphocytes from (a) Healthy donors and (b) Patients with bronchial asthma (fraction 0.4); where Io-fluorescence intensity of DNA complex-DNase (enzyme application-0 min); It-fluorescence intensity of DNA complex-DNase after t time (linear scale)

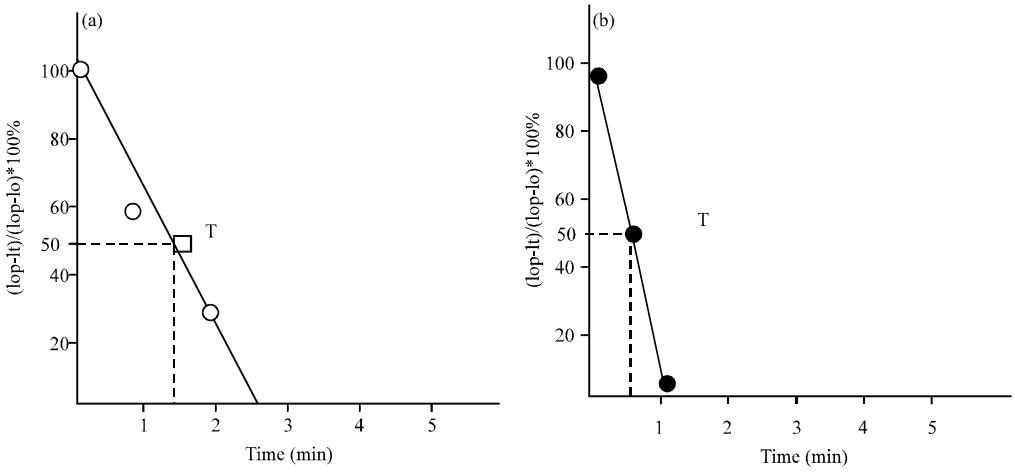


Fig. 11(a-b): Determination of $T_{1/2}$ (semilogarithmic scale); where Io-intensity of fluorescence of complex DNA-DNase (moment of enzyme application-0 min); It-intensity of fluorescence of complex DNA- DNase after t time; Iop-fluorescence under circumstances where all DNA molecules are open; $T_{1/2}$- period of time during which half of molecules of supercoiled DNA received breaks; $T_{1/2}$ for reaction of DNA with DNase of a healthy donor-1.5 min (a), $T_{1/2}$ for reaction of DNA with DNase of a patient with bronchial asthma-0.5 min (b)

healthy donors -69.17±34.93 sec, in other words $T_{1/2}$ of patients is about 23% lower than that of healthy persons. Accordingly, generation of single breaks in 50% molecules of supercoiled DNA requires from patients DNase less time than that of healthy cells DNase, which attests to a higher Mn^{2+}-dependent activity in cells from sick donors.

However, in patients the speed of DNA hydrolysis by protein-clinging enzymes differed significantly: in 6 cases the activity of DNases of patients was higher than activity of healthy donors ($T_{1/2}$ patients with BA<$T_{1/2}$ healthy donors), in 3 cases the activity of DNases of healthy donors was higher than that of patients ($T_{1/2}$ patients with BA>$T_{1/2}$ healthy donors). In this terms Dnase activity in two out from 9 patients significantly (by 3-4 times) exceeded DNase activity of healthy donor. In four cases DNase activity of cells in patients exceeded activity of DNases of cells in healthy donors for 30-50%, in other words a positive correlation was established between the activity of Mn^{2+}-dependent DNase and the stage of disease in time of course. The time of generation of single breaks in 50% DNA molecules ($T_{1/2}$) of patients made (Fig. 11) at average 0.5 min and healthy donors -1,5 min. Thus, in lymphocytes of patients two activities were revealed: Ca^{2+}, Mg^{2+}-dependent and Mn^{2+}-dependent DNase activity. In patients cells Mn^{2+}-dependent activity is higher and in healthy donors cells -Ca^{2+}, Mg^{2+}-dependent DNase activity. Especially theses differences are vivid in fractions of proteins connected with chromatin.

Chronic Obstructive Pulmonary Diseases (COPD) include a group of pulmonary diseases of heterogeneous nature-bronchial asthma, emphysema, chronic obstructive bronchitis which have a common feature: obstructive disorders of external respiration function of lungs. These diseases are among the leading reasons for rise in work incapacity and disability and take the fourth or fifth place in the rating of death causes globally. Russian medical statistics data demonstrate that respiratory diseases rank first among the most widely spread ones: 15073.2 per 100,000 people, followed by cardiovascular diseases -14385.4, nervous diseases-13491.0. (Novikov, 1996). Initial pathogenic mechanisms of COPD are at the molecular and cellular levels (Belushkin *et al*., 1998). This particular level determines the individualization of clinical implications of COPD in the ailing. While the pathogenesis of bronchi hyperactivity occurring at the first stage of the disease is not quite clear, it is assumed that it results from a complex correlation of T-lymphocytes, neutrophils, mast cells, macrophages and thrombocytes. It is clearly demonstrated that lymphocytes and other cells having migrated into the pulmonary tissue have a very limited longevity after which they get eliminated through initiation of a scheduled cell death process or apoptosis (Alles *et al*., 1991; Zhong *et al*., 1993). Scientists efforts in apoptosis studies allowed us to make within a relatively short period of time (from the beginning of 90s) important discoveries in molecular mechanisms of triggering this process (Yoshida, 2005). Significant success was achieved in the understanding of structure and functioning of Fas- receptor and molecules bound to it, factors controlling apoptosis (Bc1-2, Bax, etc) and serine proteases (caspase) (Baranovski *et al*., 2004). At the same time the nature of endonuclease responsible for DNA scission during apoptosis and the way of proteolysis produced by caspases resulting in activation of these endonucleases is not determined yet. Nevertheless the role of trigger mechanism ensuring the transition of apoptosis process to an irreversible stage, is assigned to enzyme-endonuclease.

Activation of endonuclease is followed by DNA fragmentation. This fact on its own implies death of cells unavoidably. It was established that activation of endonuclease and death of thymocytes at an early stage depend on the significant rise in cytosol of concentration Ca^{2+} the most part of which was from extracellular origin. The work (Oshimi and Miyazaki, 1995) states that lymphoid cells are characterized by a Ca-dependent way of apoptosis and calcium increase is a factor predisposing apoptosis in thymocytes (Beaver and Waring, 1995) and lymphocytes (Oshimi and Miyazaki, 1995) and these data are proved in work (Green and Scott, 1994) which reveals in human B-lymphocytes that calcium decrease restrains apoptosis of these cells. According to literature data in apoptizing cells of peripheral blood, including in T-lymphocytes and considering that rule, endonucleases activated by Ca^{2+} and Mg^{2+} ions are defined.

Thymocyte nuclei contain significant amount of Ca^{2+}, Mg^{2+}-dependent endonuclease, believed that such enzyme is activated by glucocorticoid. However, in other T-cells susceptible to apoptosis, for example, in thymoma S49 where DNA fragmentation takes place during incubation with dexamethasone, endonuclease was not defined. It is not known yet whether Ca^{2+}, Mg^{2+}-dependent endonuclease is a solitary protein (King *et al*., 1994) synthesis of which is necessary or whether it exists a number of endonucleases in T-cells which are necessary for apoptosis to start. Thus, the problem of 'apoptizing' nucleases in cells of peripheral blood remains unsolved. Our attention is called by apoptotic DNase-gamma discovered and extracted from nuclei of thymocytes in rats. Molecular mass of enzyme 33 kDa and its work requires Ca^{2+} and Mg^{2+} ions simultaneously, where such necessity is partially suppressed by Mn^{2+} (Higami *et al*., 2003) ions. Taking into account the received experimental findings concerning reduction in patients lymphocytes of Ca^{2+}, Mg^{2+}-dependent DNase activity, the appearance of Mn^{2+}-dependent DNase activity and the data from literature about the role of free calcium in lymphocytes apoptosis, we can assume that the reason of resistance to apoptosis of lymphocytes in patients with bronchial asthma is a reduction of concentration of endocellular calcium and an increase of manganese ions content, which results in triggering of activation mechanism for Mn^{2+}-dependent endonuclease activity. This leads to change of DNA fragmentation nature in lymphocytes and, as a consequence, to disorders in process of apoptic bodies' formation, thus hindering apoptosis of lymphocytes in patients with bronchial asthma.

CONCLUSION

Our research showed the role of Ca^{2+}, Mg^{2+} dependent Dnase and Mn^{2+} dependent nuclease in the apoptosis of lymphocytes in patients with bronchial asthma. These results could open the door to new perspective for in depth study of the role of apoptotic nuclease in the mechanism of asthma regulation and the target for its treatment according to the severity.

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