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The content of hypoxia-inducible factors and mediators of immunosuppression in the blood in diseases associated with hypoxia

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ABSTRACT

The aim of the study was to identify general patterns and features of changes in the content of hypoxia-inducible factors-1 and -2 in association with an imbalance of cytokines (IL-10, IL-13, galectin-2 and -9, IFN-gamma) in the blood in diseases associated with hypoxia.

Materials and methods. We examined 25 patients with coronary heart disease (CHD) with heart failure II-III according to NYHA, 16 patients with chronic obstructive pulmonary disease (COPD) without exacerbation, 16 patients with infiltrative pulmonary tuberculosis (TB) before anti-TB therapy, and 18 relatively healthy donors. Plasma concentrations of HIF-1alpha, HIF-2alpha, IL-10, IL-13, galectins-2 and -9, and IFN-gamma were determined by enzyme-linked immunosorbent assay (ELISA).

Results. Positive outcomes of quantity determination of HIF-2alpha in the blood (24.00 ± 8.54 %, 75.00 ± 10.83%, 43.75 ± 12.40% of patients, respectively, against «zero» values in healthy donors) and also signs of immunosuppression at normal plasma concentrations of HIF-1alpha were determined in diseases associated with chronic hypoxia (in patients with CHD, COPD, TB). Immunological insufficiency in CHD and TB is caused by a deficiency of IFN-gamma and galectin-2 in association with an excess of galectin-9 (in patients with CHD 1.10 [0.52; 2.60] pg/ml $p = 0.038$) or IL-13 (in patients with TB 0.81 [0.79; 1.40] pg/ml, $p = 0.043$), and in patients with COPD it is caused by a surplus of galectin-9 and IL-13 (8.50 [3.96; 15.00] pg/ml, $p = 0.001$ and 2.62 [1.20; 7.58] pg/ml, $p = 0.002$, respectively) at normal concentrations of IFN-gamma and galectin-2. The content of IL-10 in the blood tends to increase in CHD and COPD.

Conclusion. In patients with CHD, COPD and TB, chronic hypoxia is associated with immunosuppression mediated by an imbalance of IL-10, IL-13, IFN-gamma, galectins (2 and 9) in the blood and the secretion of HIF-2alpha, which has the property to stimulate the differentiation of M2-macrophages synthesizing anti-inflammatory cytokines.

Key words: hypoxia, hypoxia-inducible factor (HIF), coronary heart disease, chronic obstructive pulmonary disease, tuberculosis, interleukin, galectin, immunosuppression.

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Содержание гипоксия-индуцируемых факторов и медиаторов иммуносупрессии в крови при заболеваниях, ассоциированных с гипоксией

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РЕЗЮМЕ

Цель. Выявить общие закономерности и особенности изменений содержания гипоксия-индуцируемых факторов-1 и -2 в ассоциации с дисбалансом цитокинов (интерлейкина (IL)-10, IL-13, галектина-2 и -9, интерферона γ (IFNγ)) в крови при заболеваниях, ассоциированных с гипоксией.

Материалы и методы. Обследованы 25 пациентов с ишемической болезнью сердца (ИБС) с сердечной недостаточностью II–III по NYHA; 16 пациентов с хронической обструктивной болезнью легких (ХОБЛ) вне обострения; 16 больных с инфильтративным туберкулезом легких (ТЛ) до проведения противотуберкулезной терапии; 18 относительно здоровых доноров. В плазме крови определяли концентрацию HIF-1α, HIF-2α, IL-10 и -13, галектина-2 и -9, IFNγ методом иммуноферментного анализа.

Результаты. При заболеваниях, ассоциированных с хронической гипоксией (у больных ИБС, ХОБЛ, ТЛ), обнаруживаются положительные результаты определения HIF-1α в крови (у 24,00 ± 8,54; 75,00 ± 10,83; 43,75 ± 12,40% больных соответственно при «нулевых» значениях показателя у здоровых доноров) на фоне нормальной плазменной концентрации HIF-1α, а также признаки иммуносупрессии. Иммунологическая недостаточность при ИБС и ТЛ обусловлена дефицитом IFNγ и галектина-2 в ассоциации с избытком галектина-9 (у больных ИБС 1,10 [0,52; 2,60] пг/мл; p = 0,038) или IL-13 (у пациентов с ТЛ 0,81 [0,79; 1,40] пг/мл; p = 0,043), а у больных ХОБЛ – профицитом галектина-9 и IL-13 (8,50 [3,96; 15,00] пг/мл; p = 0,001 и 2,62 [1,20; 7,58] пг/мл; p = 0,002 соответственно) при нормальной концентрации IFNγ и галектина-2. Содержание IL-10 в крови проявляет тенденцию к увеличению при ИБС и ХОБЛ.

Заключение. У больных ИБС, ХОБЛ и ТЛ хроническая гипоксия ассоциирована с иммуносупрессией, опосредованной дисбалансом IL-10, IL-13, IFNγ, галектина-2 и -9, в крови и секрецией HIF-2α, который обладает свойством стимулировать дифференциацию М2-макрофагов, синтезирующих противовоспалительные цитокины.

Ключевые слова: гипоксия, гипоксия-индуцируемый фактор (HIF), ишемическая болезнь сердца, хроническая обструктивная болезнь легких, туберкулез, интерлейкины, галектины, иммуносупрессия.

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INTRODUCTION

In the last decade, the number of publications devoted to the study of features of intracellular signaling and processes of intercellular cooperation in cell adaptation to hypoxia has increased. Moreover, the majority of literature concerning this topic contains information on the formation of hypoxia-induced factor-1 (HIF-1) increasing under oxygen deficiency condition [1–4]. Scientists' interest in assessment of this factor in hypoxia results from the universality of this response (hypoxia refers to typical pathological processes) and, on the other hand, is due to the fact that the oxygen dependent HIF-1alpha subunit is synthesized in any nucleated cell of the body. After its interaction with the constitutive HIF-beta subunit, the transcription factor HIF-1 is formed. Then it translocates to the cell nucleus and activates the expression of glycolysis enzymes genes, anti-apoptotic factors, and proinflammatory cytokines, realizing a stress reaction, which includes a quick cell adaptation to oxygen deficiency conditions, cell protection from death, and an inflammatory response [1]. Thereby, the notion that hypoxia induces inflammation and activation of immune cells is naturally formed.

However, despite an increase in the proportion of CD4+ T-lymphocytes in chronic heart failure, of both ischemic and non-ischemic origin, these patients have interferon-gamma (IFN-gamma) deficiency [5], which suggests a qualitative inferiority of cell-mediated immune response in hypoxia. In addition, in diseases associated with hypoxia (in particular, in patients with coronary heart disease (CHD), pulmonary tuberculosis (TB) and chronic obstructive pulmonary disease (COPD)), an increase in the production of the main immunosuppressive cytokine, transforming growth factor beta (TGF-beta), has been observed. This cytokine inhibits Th1-pathway of the immune response, the synthesis of interleukin (IL)-2, as well as IL-1 and other proinflammatory cytokines, suppresses IL-2-depended proliferation of T-lymphocytes, activity of natural killers and B-lymphocytes, and production of reactive oxygen species, but it stimulates the differentiation of immunosuppressive regulatory T-lymphocytes, fibroblasts, tissue regeneration and organ fibrosis [7, 8]. Since the presence of hypoxia in CHD, COPD and TB is positive, and the TGF-beta surplus and immunosuppression and anti-inflammatory response mediated by it obviously do not correspond to the effects of HIF-1, this may be provided by the change in the production of HIF-2 also having two subunits HIF-beta and HIF-2alpha. At the same time, little is known about the modulation of the synthesis of the latter in various diseases associated with hypoxia, as well as about the production of other immunosuppressive and anti-inflammatory mediators, such as IL-10, IL-13, galectin-9, especially in combination with an imbalance of cytokines stimulating the immune system (IFN-gamma, galectin-2, etc.).

The aim of the study is to identify general patterns and features of changes in the content of hypoxia-inducible factors-1 and -2 in association with an imbalance of immunoregulatory cytokines (IL-10, IL-13, galectin-9, IFN-gamma) in the blood in diseases associated with hypoxia (coronary heart disease, chronic obstructive pulmonary disease, pulmonary tuberculosis).

MATERIAL AND METHODS

The study involved 75 people aged 45–65 years: 25 patients with CHD and postinfarction cardiosclerosis (19 men and 6 women, average age 52.18 ± 4.37 years) and 18 relatively healthy donors (12 men and 6 women, average age 49.82 ± 5.9 years) comparable in terms of gender and age with patient groups.

The criteria for including patients in the study were the presence of cyanosis, dyspnea, weakness according

to medical records analysis, as well as a decrease in the degree of saturation of hemoglobin with oxygen less than 60% and partial pressure of oxygen (p_{O_2}) in venous blood less than 37 mm Hg for patients with coronary heart disease [9], a decrease in forced expiratory volume in 1 sec (FEV_1) and Index Tiffeneau less than 75% for patients with COPD, and a decrease in FEV_1 less than 75% and lung capacity less than 85% for patients with TB, which corresponds to hypoxemia in patients with COPD and TB [9, 10]. The criteria for exclusion of patients with CHD, COPD, and TB from the study were age over 65 or younger than 45 years, the presence of hematological, tumor or autoimmune diseases, HIV infection, viral hepatitis, megaloblastic or hypoplastic anemia, acute inflammatory process at the time of the study or in 3 previous weeks before it, treatment with anti-inflammatory (steroidal and non-steroidal), immunomodulating or antibacterial agents, refusal of the study, as well as the presence of COPD or TB in patients with CHD, and the presence of CHD in patients with COPD or TB.

The material for the study was 5 ml of heparinized venous blood (25 U/ml) taken in the morning in the fasted state under aseptic conditions. The concentrations of the following cytokines IL-10, IL-13, IFN-gamma, galectin-2 and -9, HIF-1alpha and HIF-2alpha in blood plasma were determined by enzyme-linked immunosorbent assay by the aid of the following commercial kits: "IL-10-ELISA-BEST", "gamma-IFN-ELISA-BEST" produced by Vector-BEST JSC (Novosibirsk); "Human IL-13 Platinum ELISA" (eBioscience, Austria), "Human Galectin-2 ELISA Kit", "Human Galectin-9 ELISA Kit", "Human HIF-1alpha ELISA Kit" and "Human HIF-2alpha ELISA Kit" (Cloud-Clone-Corp., USA).

Statistical analysis of the results was carried out using the program Statistica 10.0 and Microsoft Excel. The median (*Me*), and the 1st and the 3rd quartiles (Q_1 and Q_3) were calculated for a statistical description; the sample fraction of the occurrence of the feature (the presence of HIF-2alpha in the blood) and its error were calculated to assess the HIF-2alpha content in the blood. The nonparametric Mann – Whitney test was used for the purpose of comparative analysis. The results were considered reliable at a statistical significance level $p < 0.05$.

RESULTS

It was shown that the content of HIF-1alpha in the blood of all examined patients corresponds to the norm (Table 1). The absence of HIF-1alpha surplus in the blood in CHD, COPD, and TB in the setting of hypoxia (see criteria for inclusion of patients in the study) can be explained by the formation of chronic hypoxia in these diseases rather than an acute one. It was shown that the content of HIF-1alpha in cells and in the blood increases in response to a rapid decrease in its oxygenation, and it normalizes after several episodes of hypoxic preconditioning of tissues [2]. HIF synthesis switches from HIF-1 to HIF-2 in chronic hypoxia: HIF-1alpha mediates a proinflammatory response and a rapid cell adaptation to acute hypoxia, and HIF-2alpha initiates a prolonged adaptive tissue response to oxygen deficiency, inducing neoangiogenesis, fibrosis, and tissue remodeling, as a consequence [1, 4].

A comparative analysis of the concentration of HIF-2alpha in the blood of patients with CHD, COPD, TB and healthy individuals was difficult because there were a large number of variants with zero value. According to the manufacturer's data (Cloud-Clone-Corp., USA) set forth in the instructions for the reagent kit for HIF-2alpha evaluation, this molecule is not determined in the blood of healthy individuals or its content is below the sensitivity limit of the kit. Therefore, a comparative analysis of the frequencies of occurrence of values other than zero in the groups of examined individuals was carried out. The study showed that positive results of HIF-2alpha determination in the blood were positive in patients with all the three types of pathology associated with hypoxia by contrast with healthy donors (Table 1). The highest number of positive results was in patients with COPD, apparently due to bronchial wall fibrosis resulting from productive inflammation, airway obstruction, and destructive changes in the lung parenchyma. The frequency of determination of HIF-2alpha in blood plasma of patients with TB was slightly lower, probably, due to the ability of intact sections of the lungs to make compensation for ventilation disorders. The lowest frequency of positive results of HIF-2alpha determination was observed in patients with CHD, which may be explained by the category of examined individuals in whom the left ventricular ejection fraction was intact, and therefore hypoxia, obviously, was of a recurring, episodic nature (increases with moderate physical exertion and practically absent at rest).

The balance between HIF-1 and HIF-2 has a significant effect on the state of the immune system. Thus, the accumulation of HIF-1 in myeloid cells promotes the synthesis of proinflammatory cytokines, while its accumulation in lymphocytes inhibits the maturation of $Foxp3^+$ T-cells (Treg) with an immunosuppressive

function [11]. An increase in the HIF-1 concentration in the medium promotes the maturation of macrophages into proinflammatory M1-cells; and an excess of HIF-2 promotes the polarization of differentiation of macrophages into anti-inflammatory M2-cells [4], which synthesize a spectrum of anti-inflammatory cytokines capable of inducing immunosuppression [12]. In view of the above mentioned, the formation of chronic hypoxia with the accumulation of HIF-2alpha in patients with CHD, COPD or TB should naturally lead to immunosuppression, which explains the excessive secretion of TGF-beta by various cells in these diseases [6, 7].

Analysis of the cytokine spectrum, namely a different combination of mediators in the blood depending on the nature of the disease, confirmed signs of immunosuppression in patients of all the three groups of study. In such a way, a high level of galectin-9 was observed in patients with CHD; an excess of IL-13 was detected in patients with TB; and both factors were presented simultaneously in individuals suffering from COPD (Table 1). Moreover, a deficiency of such immuno-stimulating factors, as IFN-gamma and galectin-2, was observed in patients with CHD and patients with TB, which was not observed in patients with COPD. The content of IL-10 in the blood in TB corresponded to norm, but it tended to elevated IL-10 in the blood in patients with CHD and patients with COPD (Table 1).

An increase in the concentration of IL-10 in the blood in CHD and COPD did not reach statistical significance of the differences compared with the group of healthy donors ($p < 0.05$), possibly, due to the small number of examined patients with COPD and the low degree of intracardiac hemodynamics disorder in CHD. In particular, a significant excess of IL-10 in the blood of patients with CHD suffering from ischemic cardiomyopathy with reduced ejection fraction of left ventricle was observed [13], as we have previously shown, which confirms the significance of the trend identified in this study. Therefore, it is possible, that IL-10 in CHD and COPD may exert its immunosuppressive effect, which involves inhibition of the cell-mediated immune response, reducing in the synthesis of IL-2, IL-3, IFN-gamma, tumor necrosis factor alpha (TNF-alpha), CD4+ T-cell migration and antigen presenting properties of macrophages and B-lymphocytes [3, 8]. The relationship between IL-10 production and hypoxia is confirmed by the presence of several HIF-1alpha motifs in the structure of its gene and stimulation of IL-10 synthesis under prolonged (22 weeks) experimental hypoxia conditions [3]. The prolonged nature of hypoxia in this experiment and data on 48% homology of HIF-1alpha and HIF-2alpha [1] suggest IL-10-stimulative effect of HIF-2alpha too, the presence of which was observed in the blood of the patients with CHD and COPD (Table 1).

Table 1

Concentration of hypoxia-inducible factors, cytokines and galectins in the blood of patients with CHD, COPD and pulmonary tuberculosis, *Me* [Q_1; Q_3]

Content of mediators in the blood	Groups of examined persons			
	CHD	COPD	PTB	Healthy donors
HIF-1alfa, ng/ml	0.052 [0.041; 0.140] $p = 0.187$	0.078 [0.026; 0.986] $p = 0.912$	0.050 [0.027; 0.092] $p = 0.203$	0.080 [0.052; 0.096]
Percentage of patients with a positive result of HIF-2alfa determination in the blood, %	24.00 ± 8.54 $p < 0.05$	75.00 ± 10.83 $p < 0.001$	43.75 ± 12.40 $p < 0.01$	0
IFN-gamma, pg/ml	0	0.60 [0; 0.87] $p = 0.364$	0	3.00 [0.50; 5.40]
IL-10, pg/ml	25.00 [23.00; 29.50] $p = 0.113$	27.50 [23.50; 31.00] $p = 0.094$	20.50 [18.50; 22.50] $p = 0.871$	19.50 [18.00; 24.00]
IL-13, pg/ml	0.62 [0.41; 0.84] $p = 0.720$	2.62 [1.20; 7.58] $p = 0.002$	0.81 [0.79; 1.40] $p = 0.043$	0.50 [0.40; 0.75]
Galectin-2, pg/ml	3.18 [2.00; 3.96] $p < 0.001$	11.00 [7.05; 12.10] $p = 0.518$	3.85 [1.55; 10.88] $p = 0.047$	13.50 [11.50; 17.00]
Galectin-9, pg/ml	1.10 [0.52; 2.60] $p = 0.038$	8.50 [3.96; 15.00] $p = 0.001$	0.50 [0; 1.00] $p = 0.419$	0.16 [0; 0.50]

Notes. CHD – coronary heart disease, COPD – chronic obstructive pulmonary disease, PTB – pulmonary tuberculosis, p – level of statistical significance of differences in indicators compared with healthy donors.

The immunosuppressive effects of galectin-9 are described in sufficient detail in the literature. In high concentrations, it induces apoptosis of activated CD4+ and CD8+ T-lymphocytes (but not Th2-cells), B-lymphocytes, monocytes, endotheliocytes; it promotes the maturation of $Foxp3^+$ T-cells in the presence of TGF-beta, and inhibits the maturation of Th17 lymphocytes in combination with IL-6; in moderate concentrations, it promotes the secretion of Th2-profile cytokines and shifts the balance of CD4+/CD8+ lymphocytes in favor of predominant subpopulation of CD4+ cells, and also activates cell adhesion, migration of neutrophils and eosinophils, dendritic cells differentiation, angiogenesis [14–17]. Galectin-9 is widespread in the human body: it is expressed in muscle, bone, lymphoid, and nervous tissues; it is found in the organs of cardiovascular, respiratory, digestive and many other systems; it is synthesized by endothelial cells, fibroblasts, macrophages, astrocytes, and also in excess by tumor cells [15]. Since fibroblasts are determined among the cells producing galectin-9, the excessive presence of this molecule in the blood in patients with CHD and COPD is probably explained by the presence of foci of fibrosis in the heart and bronchopulmonary system, respectively. In infiltrative TB, inflammation develops in an exudative manner with phenomena of destruction in the lungs. Signs of significant activation of fibroblasts and fibrosis, as a consequence, are detected only at the regenerations during the regression of infiltrative changes. This and the fact that patients with infiltrative TB were examined by us at the height of the disease explain the fact that the content of galectin-9 in their blood remained within normal limits (Table 1).

IL-13 is an anti-inflammatory and profibrotic mediator. It is synthesized by activated Th2 cells and CD8+ T-lymphocytes, according to some reports; it induces the production of TGF-beta, eotaxin mucin, activation of calcium-depended chlorine channel 1 (hCLCA1) in bronchial epithelial cells, increases the contractility of their smooth muscle cells, stimulates fibroblasts both directly and indirectly through polarization of macrophages maturation in M2 cells synthesizing TGF-beta [18]. At the same time, IL-13 activates the proliferation and differentiation of B-cells, the antigen-presenting function of macrophages, inhibits their secretion of cytokines, the synthesis of IFN-gamma by natural killer cells, and antibody-dependent cellular cytotoxicity [8]. In view of the profibrotic role of IL-13, an increase in its concentration in patients with COPD appears to be natural, since progressive fibrosis takes place in the bronchi in this pathology. In patients with CHD, fibrosis of the necrosis zone after myocardial infarction at the time of the study had already completed (a heart attack in past medical history), which likely explains the normal concentration of IL-13 in their blood.

In addition to an immunosuppressive cytokines excess in the blood, a deficiency of galectin-2 and IFN-gamma was detected in patients with CHD and TB (Table 1). The latter demonstrates the suppression of the Th1 immune response and the shift of the Th1/Th2 balance in the direction of the Th2 pathway, which is due to the biology of the pathogens in TB, mycobacteria (in order to escape from immunological surveillance of intracellular pathogens) [6], and the formation of soluble oxidized low density lipoproteins acquiring the properties of autoantigens in CHD [19]. At the same time, the negative effects of IFN-gamma deficiency for the immune system are quite obvious, since this cytokine is crucial in the implementation of the Th1 immune response, activates CD4+ and CD8+ T-lymphocytes, natural killer cells, increases the antigen-presenting properties of macrophages and their cytotoxicity, stimulates the synthesis of IL-6, IL-8, IL-15, the expression of adhesive molecules, etc. [8, 20]. The role of galectin-2 in immunity cannot be interpreted unambiguously. On the one hand, it is proinflammatory; it promotes the differentiation of M1 macrophages and inhibits the formation of M2 cells; stimulates monocytes synthesis of the proinflammatory cytokines, such as IL-6, TNFα, IL-12p40, IFNβ, and inhibits their production of TGF-beta, matrix metalloproteinases-2 and -9, vascular growth factor A (VEGF-A), and arteriogenesis, as a consequence [21]. On the other hand, galectin-2 inhibits the migration of monocytes/macrophages and the ability of the latter to activate T cells [21, 22]. In general, IFN-gamma can be characterized as a proinflammatory cytokine that activates the Th1 pathway of the adaptive immune response and cell-mediated mechanisms of the antigen (pathogen) destruction and galectin-2 can be characterized predominantly as a mediator of innate immunity. Deficiency of IFN-gamma and galectin-2 in the blood of patients with CHD and TB may be considered as a manifestation of secondary immunological deficiency, one of the mechanisms of which is the depression of maturation and the function of proinflammatory M1 macrophages with a predominance of the effects of M2 cells. It is known, that both mediators serve as triggers for the differentiation of

M1 macrophages [12, 21], and IL-13 and galectin-9 (their concentrations increased in patients with TB and CHD, respectively) serve as inducers of the formation of M2 macrophages [12, 14]. However, there was no deficiency of IFN-gamma and galectin-9 in patients with COPD, but the combined and significant accumulation of IL-13 and galectin-9 in the blood was revealed (Table 1), which is a sufficient condition for the differentiation of M2 cells. Moreover, the spectrum of cytokines secreted by M2 macrophages includes TGF-beta, IL-10, IL-13, etc. [12], an excess of which in patients with CHD, COPD, and TB was registered by us and described in the literature [6, 7]. The crucial factor in the formation of such an imbalance of cytokines in these diseases is, probably, chronic hypoxia, in which the accumulation of HIF-2 polarizes the differentiation of macrophages into M2 cells [4] and causes cytokine-mediated immunosuppression.

CONCLUSION

In diseases associated with chronic hypoxia (CHD, COPD, and TB), prolonged oxygen deficiency in the body is followed by the accumulation of HIF-2alpha in the blood at normal HIF-1alpha content. It seems that chronic hypoxia is associated with HIF-2alpha-mediated immunosuppression despite the proinflammatory effect of short-term oxygen starvation of organs and tissues and the inflammatory nature of these diseases. The latter stimulates the differentiation of macrophages into M2 cells synthesizing anti-inflammatory cytokines. Moreover, immunosuppression in CHD, COPD, and TB is realized through various combinations of mediators; in CHD and TB, it is caused by a deficiency of IFN-gamma and galectin-2 in association with an excess of galectin-9 (in patients with CHD) or IL-13 (in patients with TB); in COPD, it is caused by a combined surplus of both mediators under the conditions of normal IFN-gamma and galectin-2 levels in blood plasma. The content of IL-10 in the blood in CHD and COPD tends to increase and may be an additional factor of immunosuppression of these diseases, which requires further research.

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Chumakova S.P. – research design, analysis of literature, statistical processing of research results and their interpretation, drafting of the manuscript. Urazova O.I. – research design, material and technical support of the laboratory research, interpretation of the results, drafting of the manuscript. Vins M.V. – preparation of the biomaterial, implementation of the enzyme immunoassay method, analysisof literature . Shipulin V.M. – consulting on the research planning and interpretation of the results. Pryakhin A.S. – interaction with cardiac patients, provision of the clinical material, literature search. Bukreeva E.B. – ensuring interaction with patients, consulting on the interpretation of the results. Bulanova A.A. – interaction with patients, participation in the collection and preparation of the biomaterial, literature search. Koshel A.P. – provision of the clinical material, consulting on research planning. Novitsky V.V. – consulting on the interpretation of the results, editing of the text of the manuscript.

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