

## Імунозалежні механізми та молекулярно-генетичні особливості у хворих на системні хвороби сполучної тканини з кріоглобулінемічним синдромом

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**Вступ.** Кріоглобулінемічний синдром (КГС) — це імунозалежний процес, спричинений формуванням депозитів кріоглобулінів (КГ) в крові у дрібних або середніх судинах. Лімфотропні віруси, імунологічні та онкологічні захворювання найчастіше спричиняють розвиток КГС.

**Мета.** Вивчення імунозалежних механізмів та молекулярно-генетичних особливостей у пацієнтів із системними автоімунними хворобами (САХ) на тлі кріоглобулінемічного синдрому.

**Матеріали і методи.** Серед 380-ти хворих на САХ у 94-х (57,6 %) була ідентифікована активна фаза хронічної EBV-інфекції, а у 22,1 % пацієнта — активна фаза хронічної HSV 1/2-інфекції на основі ідентифікації ДНК вірусу методом полімеразної ланцюгової реакції (ПЛР) у трьох біосередовищах (кров, слина, зішкряб з місця ураження). Визначення експресій miR-146a та miR-155; miR-BART 13, 15 у зразках сироватки крові проводили за допомогою miRvana TM PARIS TM (Ambion, США).

**Результати.** Аналіз вмісту кріоглобулінів у цих хворих показав, що КГС було ідентифіковано в 118-ти (31,1 %) пацієнтів з середньою концентрацією КГ  $1,68 \pm 0,33$  г/л при нормі  $0,48 \pm 0,10$  г/л. У хворих на САХ з кріоглобулінемічним синдромом спостерігалось вірогідно менша експресія miR-146a, найвища експресія TLR9 на моноцитах, дещо менша — на лімфоцитах і найменша — на гранулоцитах, а також спостерігалось збільшення відносного числа Т-цитотоксичних лімфоцитів, лімфоцитів з рецептором до IL2, активованих CD HLA DR+—лімфоцитів на тлі зниження кількості NK-клітин та регуляторних супресивних CD4+/25+—клітин. Спонтанна й стимульована оксидативна здатність моноцитів у хворих з КГС мала чітко виражену тенденцію до збільшення порівняно з хворими без КГС та здоровими особами.

**Висновки.** Кріоглобуліни можуть виконувати роль своєрідного «містка» між вірусною інфекцією та автоімунними процесами. Кріоглобулінемічний синдром був ідентифікований у 31,1 % пацієнтів. Незважаючи на значну кількість досліджень, присвячених КГС, подальшого вивчення потребують особливості імунної відповіді у таких пацієнтів, оскільки створює ризики формування вторинних васкулітів на тлі САХ.

**Ключові слова:** системні автоімунні хвороби, вірус Епштейн-Барр, герпесвірус людини 1 та 2 типу, кріоглобуліни.

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reduction of NK-cells and regulatory suppressor CD4<sup>+</sup>/25<sup>+</sup>-cells was observed. The idiopathic and initiated oxidative monocyte capacity in CGS patients distinctly tended to increase, as compared to patients without CGS and normal individuals.

**Conclusions.** Cryoglobulins may act as the so-called bridge between viral infections and the autoimmune processes. CGS was diagnosed in 31.1% of patients. Despite a substantial number of studies dedicated to the cryoglobulinemic syndrome, the peculiarities of the immune reaction of such patients need further research, since they create the risks of secondary vasculitis against SAD.

**Keywords:** systemic autoimmune diseases, Epstein-Barr virus, human herpesvirus type 1 and type 2, cryoglobulins.

## Immune-related mechanisms, molecular and genetic characteristics of patients with the systemic connective tissue diseases with cryoglobulinemic syndrome

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**Introduction.** Cryoglobulinemic syndrome (CGS) is an immune-related process caused by cryoglobulins composition in the blood in small or medium vessels. Most frequently, CGS is triggered by lymphotropic viruses, immune-related and oncological diseases.

**Objectives.** Studying the immune-related mechanisms, molecular and genetic characteristics of patients with systemic autoimmune diseases (SAD) against the cryoglobulinemic syndrome.

**Methods.** Among 380 patients with SAD, in 94 (57.6%) progressing chronic EBV-infection was diagnosed, and 22.1% of patients were diagnosed with progressing chronic HSV 1/2-infection based on DNA virus identification through the polymerase chain reaction (PCR) in three biological media (blood, saliva, mucus membrane scraping).

**Results.** Analysis of the cryoglobulins in such patients showed that CGS was diagnosed in 118 (31.1%) patients with the mean concentration of CG1.68±0.33 g/l at a rate of 0.48 ±0.10g/l. The patients with the systemic connective tissue diseases with CGS demonstrated statistically lower miR-146a expression which resulted in the abnormal production of pro-inflammatory cytokines, the highest TLR9 expression on monocytes, slightly lower on lymphocytes, and the lowest on granulocytes; the increase in the relative amount of cytolytic T-lymphocytes, IL2 receptor lymphocytes, activated CD HLA DR+-lymphocytes against the

**Introduction.** Cryoglobulinemic syndrome (CGS) is an immune-related process caused by abnormal protein (cryoglobulins) composition in the blood that undergoes reversible precipitation at temperatures lower than 37° C in small or medium vessels which frequently results in systemic vasculitis [1]. Lymphotropic viruses, including herpesviruses (herpes simplex virus, cytomegaloviruses, Epstein-Barr virus), immune-related and oncological diseases are the most frequent CGS. CGS development is determined by the interaction between the genetic virus factors and the host [2]. The immune reactions causing the response to the virus replication in the immunocompetent cells resulting in the chronic immune system stimulation are of paramount concern.

Research testifies to the high EBV viral load in patients with the systemic diseases of connective tissue correlating with the disease activity regardless of the immunosuppressor therapy [2].

**Objectives:** We planned the studying of the immune-related mechanisms, molecular and genetic characteristics of patients with systemic autoimmune diseases (SAD) against cryoglobulinemic syndrome.

**Materials and methods.** The study involved 380 SAD patients (70 patients with systemic lupus erythematosus, 90 with systemic vasculitis, 120 with rheumatoid arthritis, 100 with psoriasis) hospitalized or undergoing outpatient treatment at the rheumatology department of Lviv Region Clinical Hospital and Lviv Region Clinical Diagnostic Center related to the clinical sites of the Department of Clinical Immunology and Allergology of Danylo Halytsky Lviv National Medical University. The control group involved 20 healthy individuals of the specific group and sex. DNA HSV 1/2, EBV was determined using the polymerase chain reaction (PCR) method using «Ampli Sens» (Russia) and «Rotor Geen 6000» (Corbett Research, Australia) diagnostic simultaneously in three biological media (blood serum, saliva, and scraping of the posterior pharynx mucus membrane). To define the level of specific immunoglobulins, enzyme immunoassay was conducted with the application of immunoassay analyzer Stat Fax® 303 Plus. The phenotypic characteristics of lymphocytes

were defined using the flow cytometry with the involvement of monoclonal antibodies on the Becton Dickinson (USA) cytofluorometer. MiR-146a and miR-155 expressions; miR-BART 13, 15 in blood samples were determined in several stages. At first, the general RNA was released using mirVana TM PARIS TM (Ambion, USA); afterwards, micro-RNA was determined using the real-time reverse transcription and PCR with the application of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), specific primers for each miRNA and general RNA. Quantitative real-time PCR was performed using TaqMan MicroRNA (Applied Biosystems, USA) analyses. The miRNA level was shown in conventional units (CU) using the specific formula. The amplification was carried out using 7500 Fast Real\_time PCR (Applied Biosystems, USA). The received data were analyzed using the 7500 Fast Real\_time PCR database [3, 4]. The research was carried out at the Department of General and Molecular Pathophysiology of Bogomoletz Institute of Physiology of NAS of Ukraine as part of the cooperation between universities. TLR9 study was carried out based on CD123<sup>+</sup> detection on the peripheral mononuclear blood cells through the ductal cytofluorometry using ductal cytoflow meter and «Beckton Dickenson» (USA) test kit [5].

The design of the work was agreed upon with the Commission on Bioethics at Danylo Halytsky National Medical University of Lviv (Minutes No. 5 of February 17, 2016) with a conclusion on the compliance with moral and ethical standards of bioethics according to ICH/GCP, Helsinki Declaration of Human Rights (1964), the Council of Europe Convention on Human Rights and Biomedicine (1997), as well as current legislation of Ukraine.

The descriptive statistics methods were applied to describe the initial condition of treatment groups. For quantitative indexes, the normality of data distribution within the groups was verified using the Shapiro-Wilk test. In most cases, the Gauss distribution is identified. The groups were compared using the Independent Samples t-Test or Paired T-Test. In the case of non-Gaussian distributions, the groups were compared using the Mann-Whitney test. The null hypothesis was rejected at  $p < 0.05$  [6].

**Results and discussion.** The analysis of cryoglobulins composition in such patients showed that cryoglobulinemic syndrome (CGS) was diagnosed in 118 (31.1%) patients with the mean concentration of  $CG1.68 \pm 0.33$  g/l at a rate of  $0.48 \pm 0.10$ g/l.

Since viruses are important in CGS development, we studied the connection between the cryoglobulinemia and the replicative activity of herpes simplex virus and Epstein-Barr virus based on RNA identification of the mentioned triggers in three biological fluids (blood, saliva, buccal scraping).

After the polymerase chain reaction in the blood, saliva, buccal scraping, it was found that 26 (22.1%) SAD patients with the cryoglobulinemic syndrome had DNA HSV 1/2: In one biological fluid — 9 (34.6%) cases, in two or three biological fluids — 15 (69.4%) cases. DNA of EBV was identified in 68 (57.6%) patients: in one biological fluid — 31 (45.6%) cases, in two or three biological fluids — 37 (54.4%) cases. In 24 (20.3%) patients, the DNA of HSV 1/2 and DNA of EBV was identified simultaneously.

A more detailed analysis of the DNA of herpesviruses is shown in Table 1.

According to Table 1, in SAD patients with the cryoglobulinemic syndrome, DNA of HSV 1/2

was most frequently identified in the buccal scraping (32.2) and saliva (26.3%); DNA of EBV — in the scraping of the posterior pharynx mucus membrane (51.7%), and less frequently — in the saliva (23,7%).

Thus, 94 (57.6%) SAD patients with cryoglobulinemic syndrome were diagnosed with progressing chronic EBV-infection and 22.1% were diagnosed with progressing chronic HSV 1/2-infection. Such a statement was based on the identification of DNA of the mentioned herpes viruses in three biological media. Epstein-Barr virus played the paramount role in the development of the cryoglobulinemic syndrome (57.6%) or its combination with the herpes simplex viruses (HSV1/2) — in 20.3% of cases.

MicroRNA plays an important role in the control of the immune response, miR-146a, miR-155 are the major immune system regulators [3]. Therefore, our next task was to study the level of miR-146a, -155 expression in the blood serum of patients with the systemic connective tissue disease with the cryoglobulinemic syndrome (Table 2).

According to Table 2, the level of miR-146a expression in SAD patients tended to reduce, and in patients with cryoglobulinemic syndrome, it was statistically twice as low as compared to healthy individuals and 1.7 lower as com-

Table 1

**Indices for determining qualitative DNA (%) and EBV in saliva, mucous scrapings, and blood serum of patients with systemic connective tissue diseases and the cryoglobulinemic syndrome (M ± m)**

Indices			Patients (n = 380)			
			(-) cryo. (n = 262)		(+) cryo. (n = 118)	
			DNA identification incident count			
			Abs.	%	Abs.	%
Saliva	HSV 1/2	(-) DNA	253	96.4	87	73.7
		(+) DNA	9	3.6	31	26.3
	EBV	(-) DNA	252	96.2	90	76.3
		(+) DNA	10	3.8	28	23.7
Mucous scraping	HSV 1/2	(-) DNA	255	97.3	80	67.8
		(+) DNA	7	2.7	38	32.2
	EBV	(-) DNA	248	94.7	76	31.4
		(+) DNA	34	13.0	61	51.7
Blood	HSV 1/2	(-) DNA	261	99.6	116	98.3
		(+) DNA	1	0.4	2	1.7
	EBV	(-) DNA	259	98.9	110	95.2
		(+) DNA	3	1.1	8	6.8

Table 2

**Equal miR-146a, miR-155 expressions in the blood serum of normal individuals and patients with systemic connective tissue diseases and the cryoglobulinemic syndrome (M ± m)**

Indices	Unit of measurement	Healthy individuals/ control group (n = 20)	Patients (n = 380)	
			(-) cryo (n = 262)	(+) cryo (n = 118)
miR-146a	CU: U/6	0.18	0.15	0.09*^
miR-155	CU: U/6	0.04	0.06	0.13*^

The results of the analysis in the studied groups were tested against the normality of statistical distribution indexes (U/6), they are shown in conventional units (CU).

**Remark:**

\*- p<0.05; \*\*- p<0.01 ; \*\*\*- p<0,001 statistical difference as compared to the control group  
^ - p<0.05; ^^ \*\*- p<0.01 ; ^^ ^ - p<0,001 statistical difference between patient subgroups

pared to patients without cryoglobulinemia (p<0.05). The level of miR-155 expression in SAD patients with cryoglobulinemic syndrome also increased statistically (p<0.05) by 3.25 times as compared to patients with no mentioned syndrome and healthy individuals.

Thus, patients with systemic connective tissue diseases and the cryoglobulinemic syndrome demonstrated statistically lower miR-146a expression, which resulted in the abnormal production of pro-inflammatory cytokines with the following aggravation of the chronic inflammatory process and activation of autoimmune reactions [3]. Statistical increase of miR-155 expression is frequently the reason for anti-infectious protection disturbances due to the defect of T-, B-, and dendritic cell functions, and thus, the activation of herpesvirus infection which is present in the body [7].

The next studies were dedicated to determining TLR9 expression on immunocompetent cells of SAD patients against hypercomplex-

my (Table 3). TLR is known to trigger the in-born immunity cells.

According to Table 3, TLR 9 expression on monocytes of SAD patients with the cryoglobulinemic syndrome was statistically 4 times higher (0.12 ± 0,045, p<0.05) as compared to the index in healthy individuals (0.03 ± 0.01%) and 1.5 times higher as compared to patients with no CGS (p>0.05). The expression of this receptor on lymphocytes with CGS also turned out to be 1.6 times statistically higher (1.30 ± 0.22, p<0,05) as compared to healthy individuals and 1.2 times higher as compared to patients with normal CG. Statistically, TLR 9 expression on granulocytes of CGS patients did not differ from the same index of normal individuals and patients without CGS.

Thus, SAD patients with cryoglobulinemic syndrome as compared to patients with the normal cryoglobulins composition showed the highest TLR9 expression on monocytes, some-

Table 3

**Indices of TLR 9<sup>+</sup>CD123<sup>+</sup> expression on monocytes and lymphocytes of the peripheral blood of patients with the systemic connective tissue disease with the cryoglobulinemic syndrome as compared to healthy individuals (M ± m)**

Indices	Unit of measurement	Healthy individuals/ control group (n = 20 )	Patients (n = 380)	
			cryo (-) (n = 262)	cryo (+) (n = 118)
TLR9 <sup>+</sup> CD123 <sup>+</sup>	monocytes	0.03 ± 0.01	0.08 ± 0.02*	0.12 ± 0.04*
	lymphocytes	0.80 ± 0.12	1.10 ± 0.10*	1.30 ± 0.22*
	granulocytes	0.014 ± 0.002	0.017 ± 0.003	0.019 ± 0.004

**Notes:**

\*- p<0.05; \*\*- p<0.01 ; \*\*\*- p<0,001 statistical difference as compared to the control group  
^ - p<0.05; ^^ - p<0.01 ; ^^ ^ - p<0,001 statistical difference between patient groups

what lower on lymphocytes, and the lowest on granulocytes [8].

Phagocytosis is the process of detoxification and elimination of foreign substances. The most informative indexes of phagocytosis include the following: the phagocytic index (PhI) and the oxygen-dependent digestion mechanism through the oxidative burst (OB) of monocytes and neutrophils [4]. Therefore, our next study was dedicated to the phagocytic activity of SAD patients with the cryoglobulinemic syndrome (table 4).

According to Table 4, the idiopathic and initiated absorbing capacity of neutrophils of SAD patients with cryoglobulinemic syndrome tended to reduce as compared to healthy individuals and SAD patients with the normal CG level. The absorbing capacity of monocytes ( $4.68 \pm 1.16\%$ ) was mostly affected in CGS patients as compared to healthy individuals ( $8.80 \pm 1.86\%$ ,  $p < 0.05$ ) against their reduced reserve capacity. The idiopathic oxygen-dependent processing of neutrophils was statistically higher in CGS patients ( $13.7 \pm 2.45\%$ ) as compared to patients without CGS ( $13.7 \pm 2.45\%$ ,  $p < 0.05$ ) and healthy individuals ( $7.14 \pm 2.21\%$ ,  $p < 0.05$ ). The initiated oxidative capacity of neutrophils was also statistically higher ( $99.8 \pm 3.56\%$ ,  $p < 0.05$ ) in CGS patients as compared to patients without CGS ( $87.6 \pm 5.14\%$ ). The idiopathic and initiated oxidative monocyte capacity in CGS patients showed a distinct tendency towards increas-

ing as compared to patients without CGS and healthy individuals.

Thus, patients with systemic connective tissue diseases with cryoglobulinemic syndrome showed the tendency to reducing idiopathic and initiated absorbing capacity of neutrophils and its statistical reduction among monocytes against the statistically high idiopathic and initiated oxidative activity of neutrophils as compared to patients with the normal cryoglobulinemic level [9].

At the next stage of our study, we defined major populations and sub-populations of lymphocytes in SAD patients with the cryoglobulinemic syndrome (Table 5)

According to Table 5, SAD patients with CGS show the tendency to the reduction of T-cells and T-helper cells against the statistical increase of cytolytic T-lymphocytes ( $28.9 \pm 2.33\%$ ) and statistical reduction of killer CD3+/8+-lymphocytes ( $6.01 \pm 1.22\%$ ) as compared to healthy individuals ( $20.6 \pm 2.91\%$  and  $9.77 \pm 1.49\%$  respectively,  $p < 0.05$ ). The relative amount of CD3+/8+-lymphocytes in CGS patients was 1.2 times higher as compared to patients without CGS, and the number of CD16+/56+-lymphocytes just tended to increase. In CGS patients, activated CD HLA DR+-lymphocytes and IL2 receptor lymphocytes (CD25+) tended to increase and the number of CD4+/25+ -suppressor lymphocytes was 1.5 times lower as compared to patients without CGS.

Table 4

**Indices of the phagocytic and oxidative activity of neutrophils and monocytes of the peripheral blood of healthy individuals and patients with systemic connective tissue diseases and the cryoglobulinemic syndrome (M ± m)**

Indices	Unit of measurement	Healthy individuals/control group (n = 20)	Patients (n = 380):	
			cryo (-) (n = 262)	cryo (+) (n = 118)
Idiopathic PhIN	%	5.70 ± 0.48	4.65 ± 0.36	4.93 ± 0.36
Initiated PhIN (E.Coli)	%	90.0 ± 8.34	84.0 ± 9.64	78.9 ± 4.65
Idiopathic PhIM	%	8.80 ± 1.86	6.16 ± 1.66	4.68 ± 1.16*
Initiated PhIM (E.Coli)	%	81.0 ± 8.12	76.0 ± 4.54	67.5 ± 4.33
Idiopathic OBN	%	7.14 ± 2.21	8.19 ± 1.23	13.7 ± 2.45*^
Initiated OBN (E.Coli)	%	91.5 ± 5.51	87.6 ± 5.14	99.8 ± 3.56^
Idiopathic OBM	%	4.92 ± 1.54	4.44 ± 1.86	5.72 ± 1.19
Initiated OBM (E.Coli)	%	68.7 ± 5.32	64.6 ± 7.43	69.9 ± 5.33

Notes:

\*-  $p < 0.05$ ; \*\*-  $p < 0.01$ ; \*\*\*-  $p < 0.001$  statistical difference as compared to the control group

^ -  $p < 0.05$ ; ^^ -  $p < 0.01$ ; ^^ -  $p < 0.001$  statistical difference between patient groups

Table 5

**Indices of population and sub-population structure of lymphocytes in healthy individuals and patients with the systemic connective tissue disease and the cryoglobulinemic syndrome (M ± m)**

Indices (%)	Healthy individuals/control group (n = 20)	Patients (n = 380)	
		cryo (-) (n = 262)	cryo (+) (n = 118)
		1	2
CD3 <sup>+</sup> -lymphocytes	68.3 ± 5.10	61.4 ± 4.77	58.0 ± 3.78
CD3 <sup>+</sup> /4 <sup>+</sup> -lymphocytes	36.3 ± 3.26	35.5 ± 2.32	34.1 ± 2.35
CD3 <sup>+</sup> /8 <sup>+</sup> -lymphocytes	20.6 ± 2.91	24.5 ± 3.23	28.9 ± 2.33*
CD19 <sup>+</sup> -lymphocytes	11.7 ± 2.67	13.4 ± 2.38	14.73.34
CD16 <sup>+</sup> /56 <sup>+</sup> -lymphocytes	9.77 ± 1.49	9.24 ± 2.14	10.01 ± 1.22
CD25 <sup>+</sup> -lymphocytes	9.15 ± 2.09	12.5±2.12	14.2±2.02
CD HLA DR <sup>+</sup> -lymphocytes	15.1 ± 1.17	17.3 ± 2.22	22.5±3.12
CD4 <sup>+</sup> /25 <sup>+</sup> -lymphocytes	8.29 ± 1.09	7.45±2.07	5.14 ± 1.21*

Notes:

\*- p<0.05; \*\*- p<0.01 ; \*\*\*- p<0,001 statistical difference as compared to the control group  
^ - p<0.05; ^^ - p<0.01 ; ^^ - p<0,001 statistical difference between patient subgroups

Thus, SAD patients with the cryoglobulinemic syndrome as compared to SAD patients with the normal cryoglobulins composition demonstrated the increase of the relative amount of cytolytic T-lymphocytes (1.2 times), IL2 receptor lymphocytes, activated CD HLA DR<sup>+</sup>-lymphocytes (1.4 times) against the reduction of NK-cells (1.5 times) and regulatory suppressor CD4<sup>+</sup>/25<sup>+</sup>-cells (1.5 times), however, without the statistical difference (p>0.05).

**Discussion.** Hyperproduction of a certain type of cryoglobulins is caused by viruses resulting in damage to the macrophage system by the hyperproduction which brings about the reduction of antigens and immunoglobulin clearance which includes B-cells activation and hyperproduction of specific antibodies [10]. The defects of the macrophage system cause the accumulation of cryoglobulins in circulation [11]. Today, the materials concerning the cryoglobulins involvement in the regulation of the immune response are abundant. The long-lasting antigenic stimulation of the immune system on the condition of increased

Th2-dependent cytokines (interleukine-3, -4, -5, etc.) facilitates excess immunoglobulin production. The antigenic shift of the trigger initiates the new types of antibodies. Several, even the smallest molecular modifications of the structure of these proteins, may have a dramatic impact on their physical and chemical properties and the development of cryoglobulinemic vasculitis [12].

In conclusion, cryoglobulins may act as the so-called bridge between viral infections and autoimmune processes. The capacity of such globulins to form cryoprecipitates separately or in interaction with other antigens results in the sedimentation of the latter on the vascular endothelium and the activation of the complement system. Cryoglobulinemic vasculitis is the consequence of vascular depositions of circulating immune complexes in the vessels. Despite the substantial number of studies dedicated to the cryoglobulinemic syndrome, many important aspects of the problem remain understudied.

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