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K.O. Lishchuk-Yakymovych [1],
I.H. Haiduchok [2],
K.E. Ischeykin [3],
V.V. Chopyak [1]

EFFECTIVENESS OF TREATMENT OF PATIENTS WITH SYSTEMIC AUTOIMMUNE DISEASES ON THE BACKGROUND OF THE REACTIVATION OF PERSISTENT EPSTEIN-BARR VIRUS INFECTION

Danylo Halytsky Lviv National Medical University [1]
Pekarska str., 69, Lviv, 79010, Ukraine
e-mail: office@lviv.meduniv.ua
"Lviv National Medical Institute" LLC [2]
Polishchuka str., 76, Lviv, 79000, Ukraine
e-mail: lvivmedinst@gmail.com
Ukrainian Medical Stomatological Academy [3]
Shevchenko str., 23, Poltava, 36011, Ukraine
e-mail: inostr@umsa.edu.ua
Львівський національний медичний університет імені Данила Галицького [1]
вул. Пекарська, 69, Львів, 79010, Україна
ТОВ "Львівський медичний інститут" [2]
вул. В.Поліщука, 76, Львів, 79000, Україна
Українська медична стоматологічна академія [3]
вул. Шевченка, 23, Полтава, 36011, Україна

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Ключевые слова: *системные аутоиммунные болезни, вирус Эпштейна-Барр, противовирусная терапия*

Abstract. Effectiveness of treatment of patients with systemic autoimmune diseases on the background of reactivation of persistent Epstein-Barr virus infection. Lishchuk-Yakymovych K.O., Haiduchok I.H., Ischeykin K.E., Chopyak V.V. *The article presents the study of effectiveness of inosine pranobex (IP) in patients with systemic autoimmune diseases (SAD) on the background of reactivation of persistent Epstein-Barr (EBV) infection. Among 380 patients with SAD (systemic lupus erythematosus, systemic vasculitides, rheumatoid arthritis, psoriasis), in 144 patients (37.9%) the reactivation of persistent EBV infection was detected through virus DNA identification using polymerase chain reaction (PCR) in three biological matrices (blood, saliva, scraping from the lesion site). 48 patients were receiving inosine pranobex at a dose of 50 mg/kg per day for three months. Treatment efficacy was controlled by studying the levels of expression of miR-146a, miR-155, miR EBV (BART-13 and BART-15), TLR9, the quantity of lymphocytes populations and subpopulations. After treatment, PCR results showed a decrease in viral replication in 66.7% of cases. The use of IP contributed to a significant decrease in the level of IgM, IgG specific antibodies, an increase in the level of expression of anti-inflammatory miR-146a, a decrease in the level of expression of pro-inflammatory miR-155 which may signify the strengthening of antiviral control. The study data demonstrated the decrease in the expression of miR EBV (BART-13 and BART-15) and TLR9 on the immunocompetent cells that can also be attributed to the criteria for IP effectiveness. The effectiveness of IP was also proved by the stabilization of cell mechanisms, namely the tendency to normalizing T and B cell populations, decrease in the number of natural killer cells and activated cells ($CD25^+$, $CD3^+$ $HLA\ DR^+$). On the other hand, the number of lymphocytes with suppressor activity ($CD4^+25^+$) remained significantly high mitigating autoimmune aggression. The results of the study show that the use of IP for treating the acute phase of EBV infection contributed to the decrease of replicative activity of the virus; suppressing the aggressiveness of autoimmune reactions. The decrease in the expression of miR EBV (BART-13 and BART-15) can be recommended as a criterion for the IP effectiveness; the decrease in the expression of TLR9 on immunocompetent cells –as a criterion for suppressing autoimmune reactions.*

Реферат. Ефективність лікування хворих на системні автоімунні хвороби на тлі реактивації персистуючої Епштейна-Барр вірусної інфекції. Ліщук-Якимович Х.О., Гайдучок І.Г., Іщейкін К.Є., Чопяк В.В. *У статті представлені дослідження ефективності застосування препарату інозин пранобекс (ІП) у хворих на системні автоімунні хвороби (САХ) з реактивацією персистуючої Епштейна-Барр (EBV)-інфекції. Серед 380*

хворих на САХ (системний червоний вовчак, системні васкуліти, ревматоїдний артрит, псоріаз) у 144 (37,9%) виявлено реактивацію EBV-інфекції на основі ідентифікації ДНК – вірусу методом полімеразної ланцюгової реакції (ПЛР) у трьох біосередовищах (кров, слина, зскрібок з місця ураження). 48 таких пацієнтів отримували інозин пранобекс (ІП) у дозі 50 г/кг на добу протягом трьох місяців. Контроль ефективності лікування проводили, досліджуючи рівень експресії miR-146a, miR-155, miR EBV (BART-13 і BART-15), TLR9, кількість популяцій та субпопуляцій лімфоцитів. Після лікування за даними ПЛР спостерігалося зменшення реплікації вірусу в 66,7% випадків. Застосування ІП сприяло достовірному зниженню рівня специфічних антитіл класів IgM, IgG, підвищенню рівня експресії антизапальної miR-146a, зменшенню рівня експресії прозапальної miR-155, що може вказувати на посилення противірусного контролю. Дані проведеного дослідження вказували на зменшення експресії miR EBV (BART-13 і BART-15) та TLR9 на імунокомпетентних клітинах, що також можна віднести до критеріїв ефективності ІП. На ефективність застосування ІП вказували також стабілізація клітинних механізмів, а саме тенденція до нормалізації Т- і В-клітинних популяцій, зменшення числа натуральних кілерів та активованих клітин ($CD25^+$, $CD3^+$ HLA DR^+). Натомість число лімфоцитів з супресивною активністю ($CD4^+25^+$) залишалось вірогідно підвищеним, стримуючи автоімунну агресію. Результати дослідження вказують, що застосування ІП для лікування реактивації персистуючої EBV-інфекції сприяло зниженню реплікативної активності вірусу; пригніченню агресивності автоімунних реакцій. Можна рекомендувати в якості критерію ефективності ІП зменшення експресії miR EBV (BART-13 і BART-15), а як критерій пригнічення автоімунних реакцій – зменшення експресії TLR9 на імунокомпетентних клітинах.

The average rate of Epstein-Barr virus (EBV) infection in the adult population exceeds 90% [4]. Epstein-Barr virus is known for its tropism to B cells and follicular dendritic cells; it uses its glycoprotein gp350 to bind to the CD21 receptor. However, the virus can infect many other cells in the human body, including T cells, NK cells, monocytes, endothelial cells, smooth myocytes, as well as macrophages and squamous and glandular epithelial cells. After clinical recovery, the Epstein-Barr virus remains in the body implementing a lytic cycle that involves copying viral DNA immediately with the cell's genetic material in the process of the virus's division. EBV DNA in latently infected cells can integrate into the host chromosomes, this bears the risk of not only malignant transformation but also the formation of other serious diseases, including autoimmune ones. A direct consequence of the presence of the EBV genome in lymphocytes is immune disfunction which is manifested by two contrary processes: simultaneous selective inhibition and excessive stimulation of immune system factors [1, 3, 14]. When EBV infects cells, it produces its transcription factors that complement EBNA2 antigen increasing the risk of systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, juvenile idiopathic arthritis, inflammatory bowel diseases, coeliac disease and Type 1 diabetes. The development of the disease can be divided into two stages: stage 1 – is determined by a genetic predisposition to an autoimmune disease associated with the HLA complex; stage 2 – is associated with the influence of numerous epigenetic triggers that increase the risk of the disease [2, 12].

The scientific data demonstrates a high EBV viral load in patients with systemic lupus erythematosus that correlates with the disease activity and does not depend on the use of immunosuppressive drugs [5, 14]. Therefore, the treatment of systemic autoimmune diseases (SAD) is not an easy task due to systemic damage to various organs and systems, the use of immunosuppressive therapy, etc. However, since EBV complicates the course of the SAD and directly leads to the malfunction of the immune system, the use of antiviral therapy is necessary [4, 17].

Hence, the objective of this research was to study the effectiveness of antiviral medication in patients with systemic autoimmune diseases based on the research of immune-related mechanisms and molecular genetic values.

MATERIALS AND METHODS OF RESEARCH

The study involved 380 patients with SAD (70 patients with systemic lupus erythematosus, 90 – with systemic vasculitides, 120 – with rheumatoid arthritis, 100 – with psoriasis), who underwent inpatient or outpatient treatment at the Rheumatology Division of Lviv Regional Clinical Hospital and Lviv Regional Clinical Diagnostic Center, which are among the clinical sites of the Department of Clinical Immunology and Allergology of the Danylo Halytskyi National Medical University of Lviv. The control group consisted of 20 apparently healthy individuals of the corresponding group and sex. The research was conducted in accordance with the principles of bioethics set out in the WMA Declaration of Helsinki – "Ethical principles for medical research involving human subjects" and "Universal Declaration on Bioethics and Human Rights" (UNESCO).

EBV DNA was identified through polymerase chain reaction (PCR) with the use of AmpliSens diagnosticum (Russia) on Rotor Green 6000 (Corbet Research, Australia) simultaneously in three biological matrices (blood serum, saliva and posterior pharynx mucous scraping). To determine the level of specific antibodies, the enzyme-linked immunosorbent assay was conducted with the help of Stat Fax® 303 Plus was applied. Phenotypic characte-

ristics of lymphocytes were determined through flow cytofluorimetry with the use of monoclonal antibodies on a Beckton Dickinson cytofluorimeter (USA). Expressions of MiR-146a and miR-155; miR-BART 13, 15 in serum samples were determined in stages. First, the total RNA was isolated with the help of mirVana TM PARIS TM (Ambion, USA); then miRNAs were determined using reverse transcription and real-time PCR. The reverse transcription was performed with the use of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), specific primers for each miRNA and total RNA [6]. Real-time quantitative PCR was performed using TaqMan MicroRNA assays (Applied Biosystems, USA). MiRNA level was presented in conditional units (CU) calculated using the appropriate formula. Amplification was performed using 7500 Fast Real_time PCR (Applied Biosystems, USA). The obtained data were analyzed with the use of 7500 Fast Real_time PCR database. The study was conducted at the Department of General and Molecular Pathophysiology of Bohomolets Institute of Physiology of the National Academy of Sciences of Ukraine. The study of TLR9 was performed based on CD123 detection $^{+}$ on peripheral blood mononuclear cells using flow cytofluorimetry with the use of flow cytofluorometry and Beckton Dickinson test system (USA) [16].

All above-mentioned studies were conducted before treatment and after the completion of three courses of therapy with inosine pranobex. After the completion of the treatment course, PCR was performed after 1.5-2 months in order to reduce the number of false-positive results.

The methods of descriptive statistics are used to describe the initial state of the main groups. For quantitative indicators, the normality of data distribution in groups was checked using the Shapiro-Wilk test. In the vast majority of cases, the presence of a Gaussian distribution is established. Comparison of groups is carried out by means of Student's t-test for independent samples or by means of Paired Student's t-test. In the case of a non-Gaussian distribution, the comparison of groups was carried out using the Mann-Whitney. The null hypothesis is rejected at $p<0.05$ [9].

RESULTS AND DISCUSSION

Enzyme-linked immunosorbent assay (ELISA) revealed EBV-specific antibodies (Table 2). Based on the EBV DNA identification in various biological matrices, the reactivation of persistent EBV infection was detected in 144 patients (37.9%) by polymerase chain reaction (PCR). In the remaining 236 patients (62.1%), the latent phase of EBV infection was identified based on the absence of EBV DNA. The clinical manifestations of the viral infection largely depended on the pathogen concentration, the stage of its replication, the activity of immune response to the pathogen, regimen and duration of the background therapy.

In the group of patients with SAD, 48 patients with EBV DNA (+) were selected: men – 11 (22.9%), women – 37 (77.1%) aged 18-38 (27,5±5,1 years). In addition to the background treatment, all patients with SAD on the background of the reactivation of persistent EBV infection were prescribed inosine pranobex at a dose of 50 mg/kg per day for three months as antiviral therapy. Serological, molecular genetic and immunological studies of patients with EBV DNA (+) were performed before the start of treatment and 3 months after the start of treatment. These patients did not receive any immunotropic drugs; potentially neurotoxic, hepatotoxic drugs; drugs that, according to the researcher, could affect the result of the study. The studies of specific IgG IgM, humoral and cell-mediated immunity values were performed before treatment and after the end of a three-month course of inosine pranobex. Identification of EBV DNA in three biological matrices was conducted 1.5-2 months after the end of the treatment in order to reduce the number of false-positive results.

Molecular genetic and serological studies of patients with SAD with the reactivation of persistent EBV infection conducted before and after IP treatment in addition to background therapy. EBV DNA was detected only in one matrix in 21 patients (in blood – 1; in saliva – 6; in mucous scrapings – in 14 patients). EBV DNA was detected in several biological matrices in 27 patients with SAD: 25 patients – in two biological matrices (3 patients – blood + saliva, 22 patients – saliva + mucous scraping); 2 patients – in three biological matrices (blood + saliva + mucous scarping).

After treatment with inosine pranobex, EBV DNA was detected in 16 patients with SAD; the virus was detected in several matrices only in 9 patients compared to 27 patients before treatment. As it can be seen from the data in Table 1, after IP treatment the number of patients with EBV DNA(+) generally decreased by 3.0 times, namely in the blood – by 2.0 times, in the posterior pharynx mucous scrapings – by 3.6 times, and in saliva – by 2.0 times. Thus, the viral load – and, therefore, the effectiveness of treatment with inosine pranobex – after the 3-month treatment course in patients with SAD was 66.7%.

Large amounts of antibodies are found in blood serum, extracellular fluid and other secretions providing a humoral response. At the same time, a certain amount of antibodies can be found on the surface of the membranes of macrophages and lymphocytes, which function as receptors for antigens; they also participate in the cellular immune response [4, 15].

Table 1

Results of EBV DNA identification in patients with systemic autoimmune diseases on the background of the reactivation of persistent EBV infection before and after treatment with inosine pranobex

Values	Patients with EBV DNA (+) (n = 48)	
	before treatment	after treatment
Saliva	6 (12.5 %)	3 (6.3 %)**
Mucuos scraping	36 (75.0 %)	10 (20.8 %) ***
Blood	6 (12.5 %)	3 (4.2 %)**

Notes: *– p<0,05; **– p<0,01 ; ***– p<0,001 significant difference between patients who received IP before and after treatment.

As it can be seen from the data in Table 2, the level of IgM VCA EBV decreased by 3.1 times (p<0.01) after IP treatment; however, it turned out to be 1.7 times higher compared to the control group (p<0.05). IgG VCA EBV concentration was statistically and significantly lower by 2.5 times (p<0.001) after IP treatment and 1.3 times higher compared to the control group (p<0.05). The quantity of specific IgG EBNA EBV in patients after treatment was 1.5 times lower than before treatment (p<0.05) and remained significantly high (p<0.05) compared to the control group. Therefore, the use of antiviral therapy contributed to reducing the activity of the humoral component of the immune system, which illustrated, on the one hand, the viral reduction, on the other hand – inhibition of autoantibodies production.

Table 2

The level of specific IgM VCA, IgG VCA and IgG EBNA antibodies in the blood serum of patients with reactivation of persistent EBV infection, who received IP in addition to the background therapy compared to healthy individuals (M±m)

Values		Healthy individuals (n = 20)	Patients with EBV DNA (+) (n = 48)	
			Before treatment	After treatment
IgM VCA EBV	CU	0.89±0.08	4.55±1.08**^	1.51±0.45*^
IgG VCA EBV	CU	31.2±3.45	99.9±5. 11***^	39.9±4. 23^
IgG EBNA EBV	CU	17.9±2.59	85.7±4.23*^	58.7±4.23*^

Notes: * – p<0.05; ** – p<0.01; *** – p<0.001 significant difference between groups of patients before and after treatment with IP in addition to the background therapy; ^ – p<0.05; ^^ – p<0.01; ^^^ – p<0.001 significant difference between patients, who received IP before and after treatment, and healthy individuals.

Our next task was to study the expression of $TLR9^{+}CD123^{+}$on monocytes and lymphocytes of peripheral blood, the levels of miR-146a, miR-155 and EBV (BART-13, BART-15) in blood serum of patients with systemic connective tissue diseases and reactivation of persistent EBV infection [1] before and after treatment with inosine pranobex.

The studies of expression levels of $TLR9^+CD123^+$ on monocytes, lymphocytes and granulocytes of peripheral blood of patients with systemic connective tissue diseases and reactivation of persistent EBV infection before and after treatment with inosine pranobex (Table 3). As it can be seen from the data in the Table, after treatment $TLR9^+CD123^+$ expression decreased by 1.7 times ($p<0,05$) in monocytes and 1.9 times ($p<0.01$) in lymphocytes, but it remained significantly and statistically higher ($p<0.001$) compared to the controls. In contrast, TLR9+CD123+ expression levels in granulocytes after treatment with inosine pranobex only tended to decrease without a significant difference (>0.05).

Table 3

Expression values of $TLR9^+CD123^+$ on monocytes and lymphocytes of peripheral blood of patients with systemic connective tissue diseases and reactivation of persistent EBV infection before and after treatment with inosine pranobex (M±m)

Values (%)		Healthy individuals (n = 20)	Patients with EBV DNA (+) (n=48)	
			before treatment	after treatment
$TLR9^+CD123^+$	monocytes	0.03±0.01	0.17±0.04***^^	0.10±0.05***^
	lymphocytes	0.80±0.12	3.34±0.99***^^	1.74±0.69***^^
	granulocytes	0.014±0.002	0.025±0.011	0.019±0.009

Notes: * – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$ significant difference compared to controls; ^ – $p<0.05$; ^^ **– $p<0.01$; ^^^ – $p<0.001$ significant difference between groups of patients before and after treatment.

The obtained data provide direct evidence of the participation of TLR9 in the immune response that prepares pathogen for interaction with T cells for the further development of the adaptive immune response. The infection has been proved to be one of the main factors influencing the changes in TLRs expression. TLRs expression level directly correlates with the severity of the process; in some cases it allows us to consider these receptors as early markers of infection. Depending on the nature of the pathogen, there is an increase in the expression of certain TLRs [7, 9]. In such a case, the synthesis and secretion of pro-inflammatory cytokines intensify, which leads to the development of inflammatory response with activation of all possible systems of defense against infectious agents [5, 13]. Under certain conditions, TLR9 can recognize its DNA that leads to producing autoantibodies to DNA [4, 9]. It is also known that EBV can regulate the expression of the mentioned receptors and, therefore, it is believed that it can be one of the mechanisms of autoaggression [11, 15].

Thus, the results of our studies demonstrate a significant decrease in TLR9 expression on immunocompetent cells after antiviral treatment. These data can justify the reduction of viral load, decrease of the activity of autoaggression reactions and inflammatory process.

An important function of miRNA is to control viral replication upon the infection and its resistance to antiviral factors. EBV was one of the first human viruses, in which miRNA expression was detected; MiRNAs can be used as targets for detecting the virus itself during in-situ hybridization [10].

We conducted the analysis of the values of expression levels of miR-146a, -155, miR-EBV (BART-13, BART-15) in blood serum of healthy individuals and patients with systemic connective tissue diseases and reactivation of persistent EBV infection phase before and after treatment with inosine pranobex (Table 4).

The results of the analysis in the study groups were tested for normality of statistical value distribution (U/6) and presented in conventional units (CU).

As it can be seen from the data in Table 4, after treatment with inosine pranobex, the level of expression of anti-inflammatory miR-146a significantly increased by 2.5 times ($p<0.01$), but it remained significantly lower if compared to healthy individuals ($p<0.05$), which can prove the increase of antiviral defense due to activation of the humoral component of innate immunity. The level of expression of pro-inflammatory miR-155 also significantly ($p<0.05$) decreased by 1.7 times and remained 2.3 times higher as compared to the

controls ($p<0.01$). Overexpression of miR-155 is also known to exhibit antiviral effects. As for miR EBV BART-13 i BART-15, their expression significantly ($p<0.05$) decreased by 1.7 and 1.6 times after treatment, which also confirms the effectiveness of inosine pranobex in the treatment of patients with SAD on the background of the reactivation of persistent EBV infection [5, 8].

Table 4

Levels of miR-146a, miR-155 expression in blood serum of healthy individuals and patients with systemic connective tissue diseases and reactivation of persistent EBV infection before and after treatment with inosine pranobex

Values (CU:U/6)	Healthy individuals (n = 20)	Patients with EBV DNA (+) (n =48)	
		Before treatment	After treatment
miR-146a	0.18	0.04***	0. 10*^^
miR-155	0.04	0.15***	0.09**^
BART-13*10^6	0.00(0.00. 0.01)	7.01 (0.05; 53.52)***	4.07 (0.03; 35.22) *** ^
BART-15*10^6	0.00(0.00. 0.00)	0.56 (0.01; 6.33)***	0.36 (0.008; 4.03)*** ^

Notes: * – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$ significant difference compared to the controls ^ – $p<0.05$; ^^ – $p<0.01$; ^^^ – $p<0.001$ significant difference between groups of patients before and after treatment.

Therefore, the increase in anti-inflammatory miR-146a expression, the decrease in pro-inflammatory miR-155 expression simultaneously with significantly low expression of viral miR EBV (BART-13 and BART-15) after treatment can prove the effectiveness of inosine pranobex as antiviral therapy in patients with SAD for treatment of the reactivation of persistent EBV infection.

We analyzed the patterns of lymphocyte populations and subpopulations in healthy individuals and patients with systemic connective tissue diseases and reactivation of persistent EBV infection before and after treatment with inosine pranobex (Table 5).

Table 5

Patterns of lymphocyte population and subpopulations in healthy individuals and patients with systemic connective tissue diseases and reactivation of persistent EBV infection before and after treatment with inosine pranobex (M±m)

Values (%)	Healthy individuals (n = 20)	Patients with EBV DNA (+) (n=48)	
		before treatment with IP	after treatment with IP
$CD3^+$ lymphocytes	68.3 ± 5.10	77.5 ± 5.12	72.9 ± 3.12
$CD3^+$/4 lymphocytes	36.3 ± 3.26	48.0 ± 4.64*	43.7 ± 3.05
$CD3^+/8^+$ lymphocytes	20.6 ± 3.91	29.7 ± 2.45*	24.5 ± 2.21
$CD19^+$ lymphocytes	11.7 ± 1.67	19.2 ± 2.12*	12.2 ± 2.43^
$CD16^+/56^+$ lymphocytes	9.77 ± 1.49	16.5 ± 1.67*	13.8 ± 1.89
$CD25^+$– lymphocytes	9.15 ± 2.09	20.2 ±3.29**	15.3 ±2.76*
$CD3^+$ HLA DR^+– lymphocytes	15.1 ± 1.17	34.1 ± 3.46*	20.9 ± 1.46*^^
$CD4^+/25^+$ lymphocytes	8.29 ± 1.08	18.2 ± 2.16**	19.9 ±1.96**

Notes: * – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$ significant difference compared to controls; ^ – $p<0.05$; ^^ ** – $p<0.01$; ^^^ – $p<0.001$ significant difference between subgroups of patients.

As it can be seen from the data in Table 5, after treatment with inosine pranobex the quantity of T cells and their subpopulations (T helper cells and cytotoxic T lymphocytes) tended to decrease and turned out to be slightly higher as compared to healthy individuals, but without a significant difference ($p>0.05$). The number of $CD16^+/56^+$-lymphocytes also decreased by 1.3 times after treatment, but without a significant difference ($p>0.05$). After treatment the number of $CD19^+$-lymphocytes, $CD25^+$-lymphocytes ($p<0.05$) and $CD3^+HLADR^+$– lymphocytes significantly decreased ($p<0.01$). The number of regulatory T-cells ($CD4^+/25^+$-lymphocytes) did not significantly change after treatment; however, it remained significantly higher by 2.4 times as compared to the data of healthy individuals ($p<0.01$). The results of some studies show that EBV directly attacks immune cells during activation and manages to avoid immune response control, protecting the infected cell from identification and affecting the functions of this cell. A pathogenic feature of reactivation of persistent EBV infection is predominant damage of effector cells – T cells and NK cells; Damage of T cells can trigger a more unfavorable course of autoaggression [6, 10]. Besides, there are reports that in patients with chronic EBV infection on the background of decreasing T cells, there is an increase in B cells with CD20 markers that causes the development of B-cell lymphomas [6]. There is a direct link between EBV infection and the activation of autoimmune reaction turning B and T cells into autoreactive cells [7, 8].

In our case, the quantity of B and T effector cells slightly decreased after treatment, though remained higher than in healthy individuals. Our results may demonstrate, on the one hand, the suppression of antibody- and cell-dependent autoaggression and, on the other hand, the formation of an effective antiviral cell-type immune response.

CONCLUSIONS

Thus, the use of inosine pranobex in addition to the background therapy contributed to strengthening antiviral defense, reducing the viral load by 66.7% (according to the identification of EBV DNA), reducing cell- and antibody-dependent autoaggression; stabilizing the damaged cell and humoral mechanisms of the immune system.

Conflict of interests. The authors declare no conflict of interest.

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