OPEN ACCESS
ISSN: 2835-9496
Rakityanskaya IA, et al. (2025): 891
Ameri J Clin Med Re, 2025; 5(4): 100209
DOI: 10.71010/AJCMR.2025-e209

Research Article
American Journal of Clinical and Medical Research

Alloferon vs Valacclovir. Anti-inflammatory Effect in Patients with Chronic Epstein-Barr Virus Infection

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Citation: Rakityanskaya IA, Ryabova TS, Kalashnikova AA (2025) Alloferon vs Valacclovir. Anti-inflammatory Effect in Patients with Chronic Epstein-Barr Virus Infection. Ameri J Clin Med Re: AJCMR-209.

Received Date: 17 March, 2025; **Accepted Date:** 24 March, 2025; **Published Date:** 01 April, 2025

Abstract

Research objective: The Epstein-Barr virus (EBV) is a common virus that affects about 90% of the world's population. It has been proven that it plays a role in the development of autoimmune, lymphoproliferative and malignant diseases. Once infected, the virus remains latent in the cell for life, evading the innate and adaptive immune response. Therefore, the antiviral action of the drugs should be aimed at releasing the viruses from the lytic cells. However, no antiviral drugs are currently licensed for the treatment of EBV infection. Therefore, a decision was made to develop and produce completely new antimicrobial drugs – antimicrobial peptides (AMP).

Material and methods: The study was conducted on 100 patients suffering from chronic Epstein-Barr virus infection (EBVI). The average age of patients was 38.34±1.05 years. The duration of the course of chronic EBV infection was 3.68±0.18 years. All patients underwent EBV DNA testing by PCR in saliva samples, IL-1β production in lymphocyte culture, and a subpopulation of blood monocytes. Patients were divided into two groups, according to the initial level of induced IL-1β. In each group, two treatment regimens were administered: alloferon and valacyclovir.

Results: Alloferon and valacyclovir have an inhibitory effect on the level of IL-1β production. That is, these drugs have not only an antiviral effect, but also a pronounced anti-inflammatory effect. The production of IL-1β by cells is inhibited by the number of copies of EBV DNA in the saliva sample.

Conclusion: Alloferon and valacyclovir inhibit the production of proinflammatory IL-1β in patients with EBV.

Keywords: Epstein-Barr virus infection, IL-1β, monocytes, alloferon, valacyclovir, therapy.

1. Introduction

In 1964, Epstein M.A. and his colleagues were the first to isolate tumor cells from a biopsy sample taken from a patient with Burkitt's lymphoma [1]. The biopsy tissue material was brought from Equatorial Africa (Uganda). The virus was identified in a tumor cell culture using electron microscopy by Anthony Epstein and Yvonne Bar. In 1968, a study was published that showed that blastoid cells isolated from the peripheral blood of patients with acute infectious mononucleosis (IM) had membrane antigens that were identical to those found in cells of Burkitt's lymphoma [2]. Similar results were obtained in the work of G Klein, G Pearson in 1968 [3]. In recent years, there have been many studies on the role of EBV in the development of cancer [4, 5].

The infection rate of the population with EBV is about 90% of the world's inhabitants. For a long time, it has been accepted to divide the virus into two types. This division is based on the sequence of alleles in the EBNA2 and EBNA-3A, -3B and -3C14 genes: 1st and 2nd (type A and type B) [6]. Transformation of B cells into lymphoblastoid cell lines is largely carried out by type 1 EBV [7]. In Europe, North and South America and Asia, type 1 is more common, and type 2 is more common in New Guinea, Central Africa and Alaska [8].

Following infection, EBV persists in the infected B cell in a latent state as a circular episome. In this state, it expresses a small number of viral genes and non-coding RNAs. During cell division, the virus enters the lytic replication phase. In this phase, most of the viral genes are expressed. After that, the viral genomes replicate and infectious virions are formed [9]. The cell then dies and releases viral particles that infect healthy cells of another host. Thus, the number of latently infected cells increases. Once infected, the virus remains latent in the cell for life, evading the innate and adaptive immune response [10]. Thus, the antiviral mechanism of the drug should be aimed at the release of virions from lytically active cells.

The ability to inhibit the lytic phase of EBV replication is possessed by such licensed drugs as nucleoside analogues (acyclovir (ACV), penciclovir (PCV), ganciclovir (GCV) and their oral prodrugs: valacyclovir (VACV), famciclovir (FAM) and valganciclovir (VGCV)), nucleotide (cidofovir (CDV) and pyrophosphate (foscavir (foscarnet sodium, PFA)) analogues [11, 12]. Although these drugs have been shown to inhibit EBV replication in vitro under experimental conditions, none of these drugs have received FDA (Food and Drug Administration) or EMA (European Medicines Agency) approval for the treatment of EBV infection [13, 14]. In 2016, De Paor M. et al published the results of an analysis of the effectiveness of infectious mononucleosis therapy for the period from 1981 to 2016 according to the WHO World Registry of Clinical Trials [15].

The analysis showed that while taking valganciclovir, the content of virus-infected lymphocytes in the peripheral blood does not decrease, but the number of EBV DNA copies in blood mononuclear cells decreases during the therapy period. After the end of taking valganciclovir, the number of EBV DNA copies returns to the original level. Gershburg E. and Pagano J.S. examined why antiviral medications did not have a significant impact on EBV infection. They concluded that the reasons for the ineffectiveness of antiviral treatment lay in the complex nature of the disease [16]:

1. A long incubation period, which can be from 4 to 6 weeks and late diagnosis;
2. The release ((«shedding») of the virus resumes within 3 weeks after stopping the drug.

The clinical symptoms of EBV are a cell-mediated immune response to infection of B cells in the peripheral blood.
Resistance to antimicrobial and antiviral drugs is growing worldwide. In this regard, in their reports, the Infectious Diseases Society of America and the European Centre for Disease Prevention and Control/European Medicines have highlighted that there are few drugs currently in development that may have advantages over the existing medications on the market [17, 18]. As a result of the discussion, a decision was made to develop and produce completely new antimicrobial drugs – antimicrobial peptides (AMP), which belong to the group of oligopeptides with different amounts (from 5 to 100 or more) of amino acids. This term is used in relation to antibiotics, antiviral and antimalarial drugs [19]. In 1939, Dubos R.J. described the first antimicrobial peptide (AMP), which was obtained from a soil strain of Bacillus [20]. This aerobic bacterium had the ability to lyse living cells of gram-positive microorganisms. In an experiment on mice, the effectiveness of AMP against pneumococcal infection was demonstrated. In 1940, Hotchkiss R.D., Dubos R.J. obtained a fraction that they called gramicidin, when separating this extract [21]. An animal-derived AMP, defensin, was obtained from rabbit leukocytes [22]. AMP has the ability to integrate into the viral envelope, cause instability of the host cell membrane by binding to heparan sulfate membrane receptors, and localize in the cytoplasm and organelles [23, 24]. The interaction of AMP with the virion capsid leads to the development of decapsidation, that is, viral DNA or RNA cannot be released and transcribed [25]. AMPs are able to induce:

1) the expression of toll-like receptors when interacting with viral nucleic acid;
2) the production of cytokines;
3) the expression of major histocompatibility complex molecules in infected cells [26];
4) activate innate restriction factors encoded by the infected cell [27].

Two variants of antimicrobial peptides, called alloferons, were isolated from bacteria-infected larvae of the blowfly Calliphora vicina. Alloferon molecules are not strictly homologous to other peptides. Two types of amino acid sequences (13 and 12 amino acids) were isolated. A peptide consisting of 13 amino acids was named alloferon 1 and in animal experiments it showed the ability to enhance antiviral and antitumor activity, induce cytotoxicity of NK cells in peripheral blood and the synthesis of interferon [28]. Alloferon is not cytotoxic, immunogenic, carcinogenic, embryotoxic and has no reproductive effects [29]. The name of the AMP "alloferon" was taken due to the identity of the function with interferon and the origin of different species of invertebrates (Allo) [30].

The aim of this study is to investigate the effect of alloferon and valacyclovir on the production of IL-1β in lymphocyte culture in patients with chronic Epstein-Barr virus infection.

2. Material and methods. Patients

This study was carried out in the Department of Allergology, Immunology and Clinical Transfusiology of Municipal Outpatient Hospital no. 112 (Saint Petersburg, Russia) from May 2022 to September 2024. The single-center cohort prospective study included 100 patients suffering from chronic Epstein-Barr virus infection (70 women and 30 men). The average age of patients was 38.34±1.05 years (95% CI: 36.20 - 40.46). The duration of the course of chronic EBV infection was 3.68±0.18 years (95% CI: 3.32 - 4.10). The general practitioner identified EBV infection during a laboratory examination, and subsequently referred the patients to an immunologist for a course of antiviral treatment.

Participants in the study were selected according to the inclusion criteria:
• age 25–50 years;
• presence of clinical complaints caused by EBV;
• EBV DNA copy number in saliva $>10^3$;
• signed voluntary informed consent before the study.
Exclusion criteria were:
• presence of autoimmune diseases associated with EBV infection;
•presence of other viral infections (cytomegalovirus infection, viral hepatitis, human immunodeficiency virus);
• presence of toxoplasmosis, borreliosis, staphylococcal and streptococcal infection;
• presence of helminthic invasions, parasitic infections;
• presence of pregnancy in women;
• presence of acute inflammatory diseases of any organs;
• presence of mental illness;
• regular alcohol consumption;
• taking any antiviral drugs in the last 6 months.

The clinical study was conducted in compliance with the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" (2013), the Protocol to the Council of Europe Convention on Human Rights and Biomedicine of 1999, Articles 20, 22, 23 of the Federal Law "On the Fundamentals of Health Protection of Citizens in the Russian Federation" dated November 21, 2011 No. 323-FZ (as amended on May 26, 2021) and was approved by the local Ethics Committee of the St. Petersburg Dialysis Center LLC FRESENIUS MEDICAL CARE (protocol No. 15 dated 12.14.2021).

The medical assessment involved gathering information about the patient's medical history, including any previous immuno- or antiviral treatments they had received, as well as any underlying health conditions they may have.

Detection of viral DNA by PCR in saliva samples. The study of the number of EBV DNA copies was carried out in saliva samples using the polymerase chain reaction (PCR) method with real-time hybridization-fluorescence detection. The test systems "AmpliSens EBV/CMV/HHV6-screen-FL" of the Central Research Institute of Epidemiology (Russia) were used. To measure the amount of DNA in saliva, we use the number of

copies of Epstein-Barr virus (EBV) DNA in 1 ml of the sample (cDNA). This indicator is calculated using the formula: CDNA = CDNA x 100, where CDNA is the number of copies of viral DNA in the sample. The analytical sensitivity of the test system is 400 copies/ml (according to the manufacturer's instructions).

Production of interleukin-1β (IL-1β) in the culture medium (spontaneous, induced production) and in blood serum. Peripheral blood was collected from the cubital vein into a vacutainer with a coagulation activator (to obtain serum) and into a vacutainer with lithium heparin as an anticoagulant (for cultural studies). To obtain serum, the blood was centrifuged for 10 minutes at 3000 rpm. To obtain cytokines, peripheral blood cells were stimulated with pyrogenal (N.F. Gamaleya National Research Center for Epidemiology and Microbiology, Ministry of Health of the Russian Federation). Pyrogenal was added at a working dilution of 10 µg/ml per well and incubated with whole blood for 24 hours in the presence of CO2 at 37°C. The content of IL-1β in serum and supernatant was determined by solid-phase enzyme-linked immunosorbent assay using the Interleukin-1 beta-IFA-Best test systems (Vector Best, Russia).

Determination of the relative number of monocyte subpopulations. Peripheral blood was collected from the cubital vein, EDTA was used as an anticoagulant. The relative number of monocyte subpopulations was estimated by flow cytometry. Whole blood cells were stained with monoclonal antibodies: CD14PE, CD16PC5, CD45APC-AF750. Depending on the level of CD14 and CD16 expression, monocytes can be divided into three subpopulations:

1. $CD14^{+}CD16^{-}$ - «Classical monocytes»;
2. $CD14^{+}CD16^{+}$ - «Transitional monocytes»;
3. $CD14^{+dim}CD16^{+}$ - «Non-classical monocytes».

Statistics. For statistical processing of the obtained data, the IBM SPSS Statistics software package, version 26 (Armonk, NY: IBM Corp.) was used. Group results are presented as the arithmetic mean M ± standard error. Parametric (Pearson's method) and nonparametric (Spearman's method, Kendall's tau(τ)) methods were used in the work. Compliance with the condition of observation independence was also checked. For this purpose, linear regression analysis (with calculation of the determination coefficient (R-square) and the Durban-Watson criterion) and variance analysis (ANOVA Analysis of Variance) with calculation of the Fisher criterion (F) for checking the significance of the model were used. The standardized ß indicator was calculated with 95% confidence intervals. The critical level of significance of the difference in indicators was equal to 0.05.

3. Results

All patients (n=100) underwent PCR analysis of EBV DNA copy number in saliva samples. The EBV DNA copy number was 189030.20 ± 23658.81 copies/ml (95% CI: 143753.24 – 236385.11) (Fig.1).

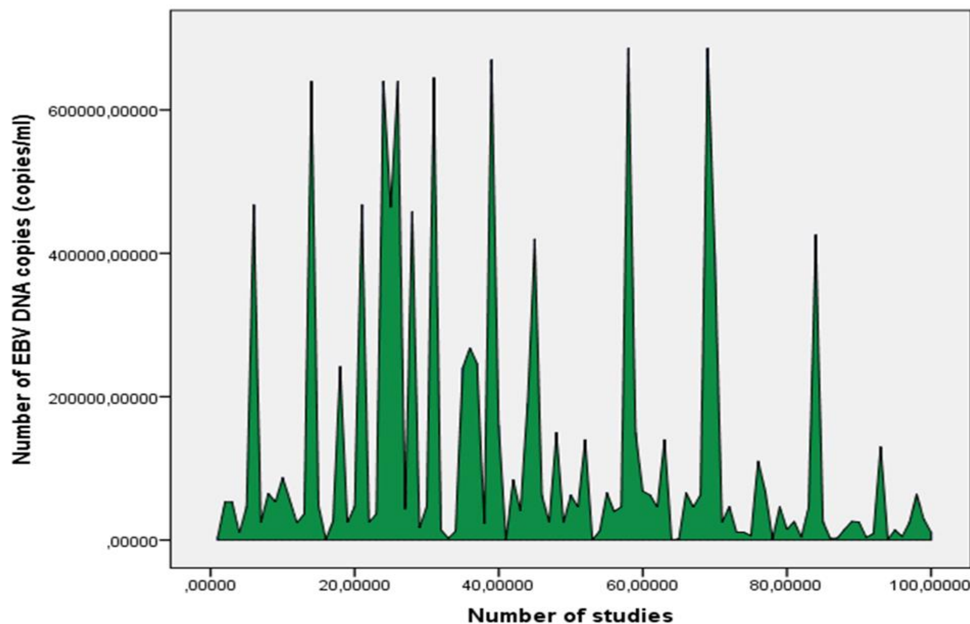

Figure 1: Number of EBV DNA copies in saliva sample from patients in the general EBV infection group before the start of therapy.

Clinical complaints. Chronic Epstein-Barr virus infection is characterized by a long course of mononucleosis-like symptoms and frequent relapses of the clinical and laboratory picture of the viral infection [31, 32]. Patients complain of prolonged subfebrile temperature (37.1-37.3°), severe weakness and physical fatigue, increased sweating (especially at night), lymphadenitis (cervical and submandibular lymph nodes, less often axillary and inguinal), pain in the throat. Frequent colds, complaints from the mucous membranes of the mouth (exacerbation of aphthous stomatitis, burning of the mucous membrane of the tongue, postnasal drip); development of arthralgia of large and stiffness of small joints may also be present. Patients have neurological complaints: headaches, decreased concentration, memory impairment, sleep disorders, irritability, tearfulness. The patients attended sessions with a psychologist, are observed by psychotherapists, and are receiving antidepressant therapy, which does not reduce the severity of the complaints described. It is noteworthy that the occurrence of the complaints described was aggravated by long-term stressful situations and psycho-emotional overloads. We analyzed the frequency of clinical complaints in the general group of patients (Table 1).

Table 1: Frequency of clinical complaints in patients with chronic Epstein-Barr infection in patients in the general group (n=100)

Clinical complaints	Frequency of clinical complaints (%)
Subfebrile temperature	91
Lymphadenitis	76
Sore throat	85
Postnasal drip	67
Stomatitis	40
Weakness	75
Physical fatigue	65
Chills	60
Sweating	85
Irritability and tearfulness	60
Headaches	37
Decreased concentration	48
Decreased memory	47
Sleep disturbance	45

IL-1β production in lymphocyte culture. In the general group of patients, a study of IL-1β production (serum, spontaneous, induced) was conducted (Table 2).

Table 2

IL-1β production in culture medium in patients with EBV infection in the general group (n=100)

Parameter	IL-1 level (pg/ml)	Reference values (pg/ml)
Serum level	4,97±1,05 95% CI: 3,14 – 7,31	0 - 11
Spontaneous level	14,04±2,64 95% CI: 9,24 – 19,33	1 - 107
Induced level	1981,24±151,48 95% CI: 1728,04 – 2304,86	50 - 1200

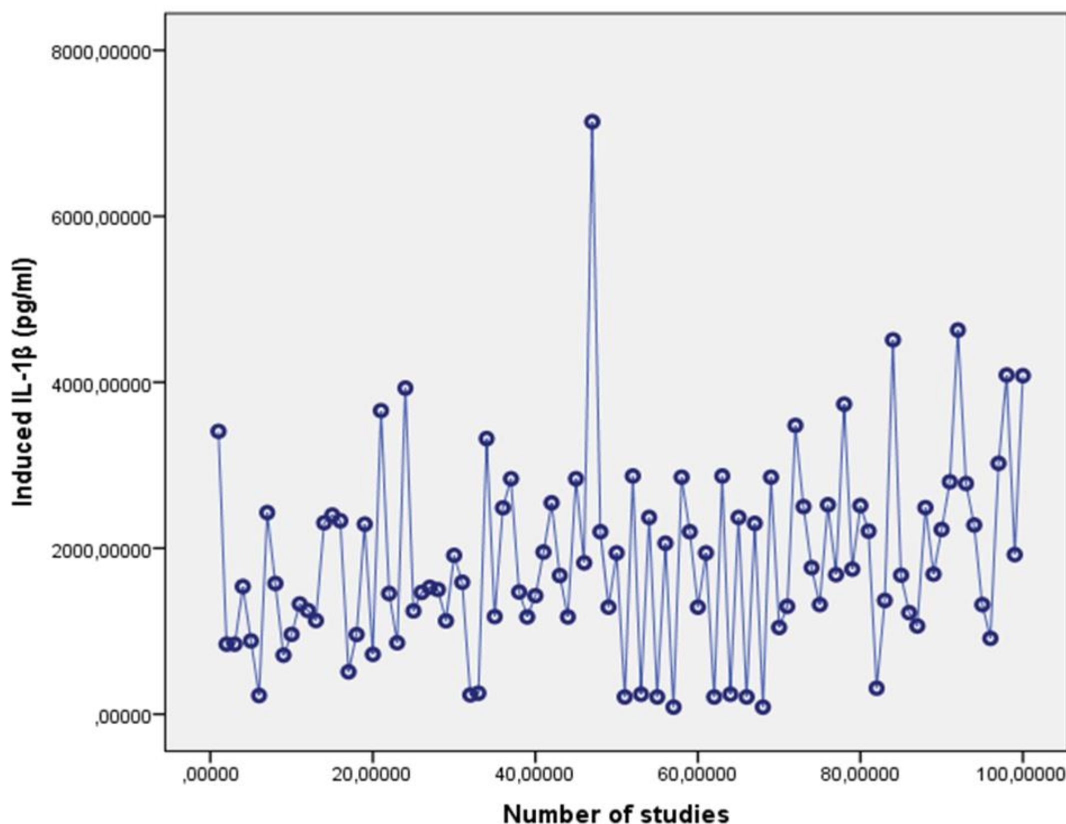

Figure 2: Level of induced IL-1β production in the culture medium of patients with EBV infection in the general group.

When we analysed the level of induced interleukin-1β in the culture medium, we found that the levels of production varied between patients, i.e. they were either within the reference values or significantly higher than the indicators (50 - 1200 pg/ml, reference values provided by the manufacturer of the test systems). Therefore, all patients in the general group were divided into two groups according to the baseline level of induced IL-1β:

- Group 1 (n = 29) - the level of induced IL-1β is normal within the range of 86 – 1176; M±м = 700.21 ± 61.06 (pg/ml) (95% CI: 574.93 - 821.12).
- Group 2 (n = 71) - the level of induced IL-1β is high within the range of -1328 – 7139; M±м = 2561.07 ± 202.95 (pg/ml) (95% CI: 2205.13 - 2991.49).

Table 3 provides a comparative analysis of these two groups of patients based on their age, the duration of the disease, and the number of EBV DNA copies found in their saliva samples.

Table 3: Comparative analysis of the 1st and 2nd groups of patients with chronic EBV infection

	Group 1 (*n* =29)	Group 2 (*n* =71)	P
Age of patients (years)	37,22±1,80 95% CI: 33,70 - 40,99	38,56±1,50 95% CI: 35,69 – 41,59	P =0,07
Duration of disease (years)	3,81± 0,33 95% CI: 3,20 - 4,50	3,62±0,29 95% CI: 3,08 - 4,22	P =0,06
Number of EBV DNA copies (copies/ml)	149805,14± 26646,49 95% CI: 27152,94- 135132,04	134371,54±24474,76 95% CI: 92079,18 - 187938,15	P =0,05

The data in the table show that patients in these groups did not differ in age and duration of the disease. However, the number of EBV DNA copies was significantly higher in patients with normal levels of induced IL-1β. At the next stage, we analyzed the content of monocyte subpopulations in the peripheral blood of patients in the general group, in the 1st and in the 2nd (Table 4).

Table 4: The percentage of monocytes in the peripheral blood of patients in the 1st and 2nd groups of EBV infection

Monocyte subpopulations/ reference values (%)	General group (*n* =100) (1)	Group 1 (*n*=29) (2)	Group 2 (*n* =71) (3)	P
Classical monocytes (CD14+CD16-) / (81,00 – 90,00)	80,84±0.59 95% CI: 79,66 - 82,06	79,53±1.12 95% CI: 77,59 -81,89	87,26±0,96 95% CI: 85,31 -89,07	P1,2= 0.08 P1,3=0.04 P2,3=0.02
Transitional monocytes (CD14+CD16+) / (5.00 – 13,00)	4.43±0,14 95% CI: 4,16 - 4,74	4,71±0,33 95% CI: 4,14 - 5,44	5,29±0,44 95% CI: 4,44 - 6,22	P1,2=0,058 P1,3=0,04 P2,3=0,05
Non-classical monocytes (CD14+dimCD16+) / (2,00 – 7.00)	6,05±0, 22 95% CI: 5,62 - 6,49	5,98±0.34 95% CI: 5,28 -6,63	6,85±0, 81 95% CI: 5,35 –8,57	P1,2 =0,08 P1,3=0,057 P2,3=0,05

The data in the table show that the content of monocyte subpopulations in the general group did not differ from the reference values. However, in the 2nd group, the content of all monocyte subpopulations in the peripheral blood was higher when compared with the 1st. Nevertheless, the content of monocyte subpopulations in both groups was within the reference values. The severity of clinical complaints in both groups of patients was approximately the same (Table 5).

Table 5: Frequency of occurrence of the main clinical complaints (%) in patients with EBV infection in both groups.

Clinical complaints	Group 1 (*n* =29)	Group 2 (*n* =71)	p
Subfebrile temperature	89,65	94,366	0,01
Lymphadenitis	62,06	73,24	0,001
Sore throat	79,31	84,50	0,01
Weakness	79,65	81,69	0,064
Chills	82,75	88,73	0,01
Physical fatigue	86,20	88,73	0,062
Sweating	68,96	70,42	0,07
Postnasal drip	89,65	81,69	0,01
Stomatitis	75,86	60,56	0,001
Irritability and tearfulness	68,96	84,50	0,001
Headaches	75,86	80,27	0,04
Decreased concentration	42,85	42,25	0,082
Decreased memory	42,85	42,25	0,082
Sleep disturbance	68.96	81,27	0,001

Based on the presented data, it can be concluded that there were no significant differences in the clinical picture between patients from different groups.

Prognostic significance of the level of induced IL-1β production. Next, we analyzed the factors that influenced the level of IL-1β production. For this purpose, we performed linear and exponential regression analysis and ANOVA "Analysis of Variance" with the calculation of Fisher's criterion (F) to test the significance of the model. The results obtained are presented below (Table 6, Figures 3, 4).

Table 6: Regression models of the dependence of the level of induced IL-1β on other factors in patients in the general group of EBV.

Indicator	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	P
Age of patients (years)	Linear	0,523	108,57	0,723	0,000
	Exponential	0,906	951,41	0,860	0,000
Duration of disease (years)	Linear	0,386	61,69	0,622	0,000
	Exponential	0,736	273,61	0,760	0,000
Number of EBV DNA copies	Linear	0,308	44,10	0.555	0,000
	Exponential	0,406	67,77	0,637	0,000
Classical monocytes (CD14+CD16-)	Linear	0,614	152,51	0,783	0,000
	Exponential	0,984	583,32	0,992	0,000
Transitional monocytes (CD14+CD16+)	Linear	0,570	127,14	0,755	0,000
	Exponential	0,897	834,14	0,947	0,000
Non-classical monocytes (CD14+dimCD16+)	Linear	0,549	116,69	0,741	0,000
	Exponential	0,872	655,80	0,934	0,000

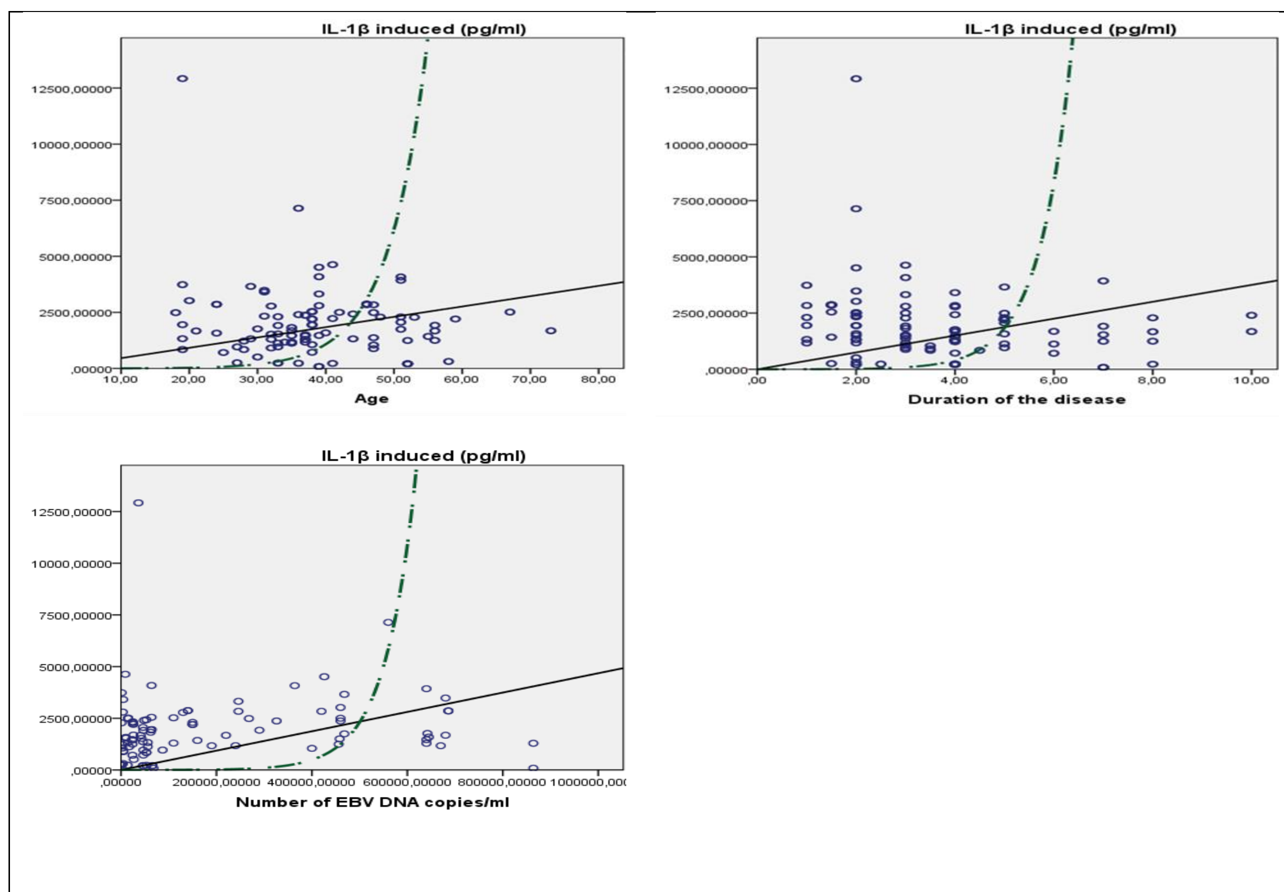

Figure 3: Dependence of induced IL-1β production on age, duration of disease, and the number of EBV DNA copies in saliva samples (·— exponential regression; — linear regression).

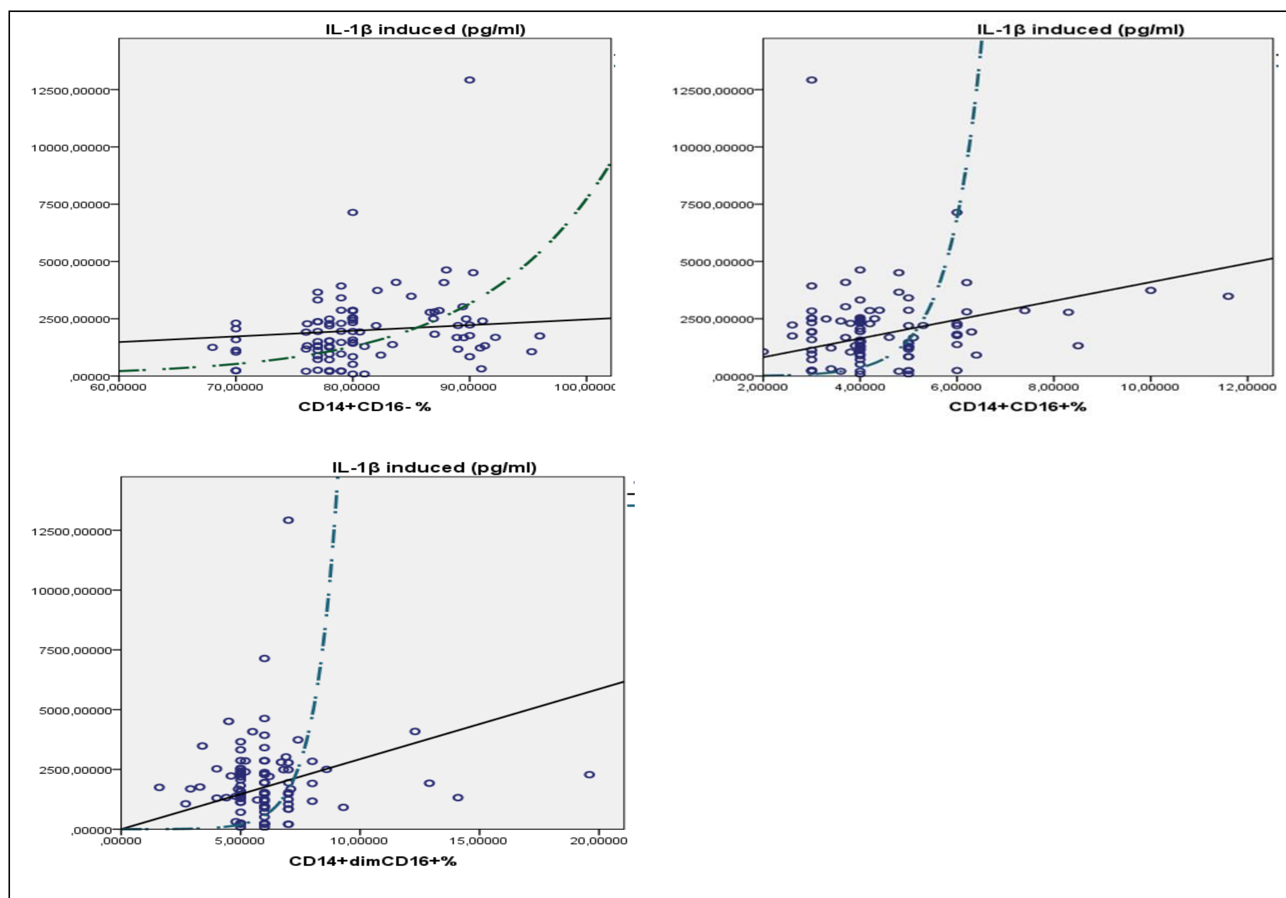

Figure 4; Dependence of induced IL-1β production on monocyte subpopulations in the peripheral blood of patients with EBV (·— exponential regression; ─ linear regression).

The presented data show that the production of induced IL-1β depends on age, duration of the disease, the number of EBV DNA copies and the content of monocyte subpopulations in the peripheral blood of patients.

Comparative analysis of the effectiveness of therapy with alloferon and valacyclovir in the 1st and 2nd groups of patients. Each group was then divided into two subgroups to receive different treatment regimens – alloferon and valacyclovir (control). Alloferon therapy was prescribed to patients who had previously received multiple courses of antiviral therapy with a drug from the acyclic nucleoside group – valacyclovir and who had developed strong resistance to this drug. Valacyclovir therapy was prescribed to patients who had not previously received any antiviral therapy.

In subgroups 1.1 and 2.1, patients received Alloferon - 12 subcutaneous injections of 1.0 mg every other day.
In subgroups 1.2 and 2.2, patients received a prolonged therapy regimen with a drug from the acyclic nucleoside group - valacyclovir (Valtrex) (500 mg x 3 times a day, orally) for two months. Four weeks after the course of therapy, patients were re-examined (Figure 5).

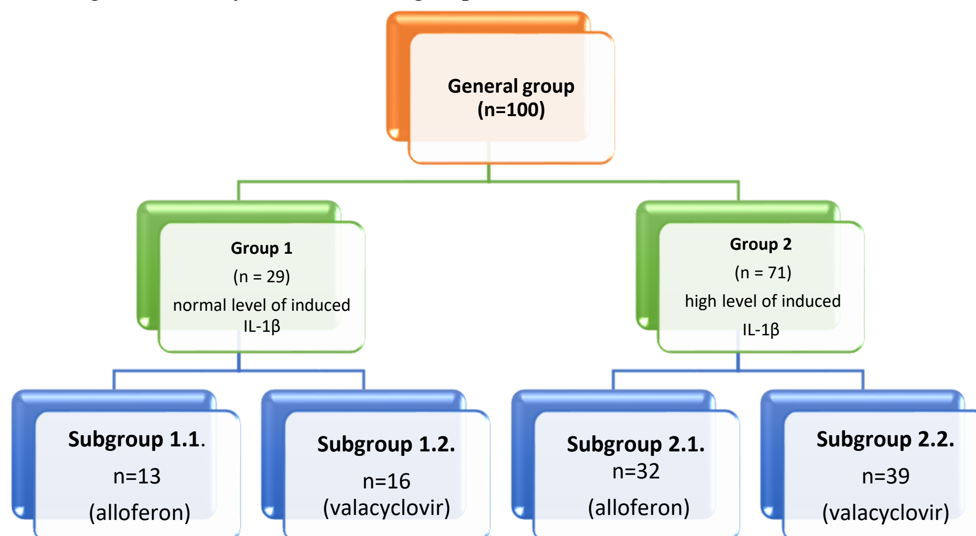

Figure 5: Scheme of distribution of patients by types of therapy

Below are the data of comparative analysis of these two subgroups with normal levels of induced IL-1β production before the start of therapy (Table 7).

Table 7: Dynamics of induced IL-1β production in subgroups 1.1. and 1.2. 4 weeks after the course of therapy.

Parameter	***Subgroup 1.1. (n=13)***		**P**
	Before Alloferon therapy	***4 weeks after completion of Alloferon therapy***	
Serum IL-1β	2,07±0,35 95 % CI: 1,46 – 2,76	1,69±0,36 95 % CI:1,07 – 2,46	0,001
Spontaneous IL-1β	3,69±1,52 95 % CI: 1,53 – 7,15	3,15±0,77 95 % CI: 1,76 – 4,76	0,002
Induced IL-1β	753,00±49,33 95%CI: 657,69 -854,13	564,69 ± 60,94 95%CI: 440,00 -684,72	0,002
Parameter	**Subgroup 1.2. (n=16)**		**P**
	Before valacyclovir therapy	***4 weeks after completion of valacyclovir therapy***	
Serum IL-1β	4,15±1,84 95 % CI: 1,73 – 8,21	2,10±0,32 95 % CI: 1,52 – 2,70	0,000
Spontaneous IL-1β	3,74±0,83 95 % CI: 2,36 – 5,63	2,89±0,40 95 % CI: 2,15 -3,68	0,000
Induced IL-1β	637,73±92,78 95%CI:461.13– 23.56	590±60,11 95% CI: 467,89 – 707,34	0,000

The data in the table show that alloferon and valacyclovir significantly reduce the level of production of serum, spontaneous and induced IL-1β in patients in both subgroups. However, all results do not go beyond the reference values, remaining within the normal range.

Comparative analysis of the effectiveness of therapy with alloferon and valacyclovir in the 2nd group with a high level of induced IL-1β. Further, a similar analysis was conducted in the 2nd group (n = 71). All patients were divided into subgroups 2.1. and 2.2. Group 2.1. (n = 32) received therapy with alloferon, group 2.2. (n = 39) received a therapy regimen with valacyclovir for two months (table 8).

Table 8: Dynamics of induced IL-1β production in subgroups 2.1. and 2.2. 4 weeks after the course of therapy

Parameter	**Subgroup 2.1. (n=32)**		**P**
	Before Alloferon therapy	***4 weeks after completion of Alloferon therapy***	
Serum IL-1β	3,35±1,33 95 % CI: 1,61 – 6,48	2,29±0,40 95 % CI:1,58 – 3,19	0,000
Spontaneous IL-1β	4,25±0,45 95 % CI: 3,35 – 5,19	3,29±0,39 95 % CI: 2,51 – 4,09	0,000
Induced IL-1β	2652,56±397,77 95%CI: 2016,33 – 3541,88	1875,20±145,23 95%CI:1624,26-2180,24	0,000
Parameter	**Subgroup 2.2. (n=39)**		**P**
	Before valacyclovir therapy	***4 weeks after completion of valacyclovir therapy***	
Serum IL-1β	2,18±0,29 95 % CI: 1,59 – 2,71	2,10±0,34 95 % CI: 1,51 – 2,89	0,004
Spontaneous IL-1β	6,25±0,95 95 % CI: 4,51 – 8,20	3.69±0,56 95 % CI: 2,71 – 4,84	0,000
Induced IL-1β	2581,76±301,75 95%CI: 2099,26 – 3248,75	1911,35 ± 189,56 95%CI:1575,24 -2317,95	0,000

The analysis of the results shows that treatment with alloferon and valacyclovir effectively reduces the production of serum, spontaneous and induced IL-1β 4 weeks after the end of the course of therapy. The maximum reduction effect after alloferon therapy was observed in patients of the 2nd group with an initially high level of IL-1β production (by 29.31% from the initial level). After a course of valacyclovir, the reduction in IL-1β production was also maximum in the 2nd group of patients (25.9703% from the initial level).

Dynamics of the number of EBV DNA copies 4 weeks after the end of therapy. Next, an analysis of the effectiveness of alloferon and valacyclovir therapy on the level of EBV DNA copies in each individual group of patients was conducted. The results are presented in Table 9.

Table 9: Dynamics of the number of EBV DNA copies in each subgroup in patients with chronic EBV infection after therapy with alloferon and valacyclovir.

Number of EBV DNA copies in saliva samples (copies/ml)		
Before alloferon therapy	***4 weeks after the end of alloferon therapy***	**P**
1.1. group (n =13)		
186690,47±49925,23 95% CI: 96765,50 -288885,87	6434,79±3137,18 95% CI: 1078,84 - 14092,63	0,000
2.1.group (n =32)		
286494,44 ±45642,20 95%CI:204312,65 - 382880,25	3455,38±2333,58 95%CI:640,62 -8584,18	0,000
Before therapy with valacyclovir	***4 weeks after the end of valaciclovir therapy***	**P**
1.2. group (n =16)		
192046,19±39941,07 95%CI:119028,47 - 269652,04	33879,20 ± 6785,58 95% CI: 7913,15 - 68859,78	0,000
2.2. group (n =39)		
232878,50±51523,76 95% CI: 136164,73 -337278,70	33879,20±15432,40 95% CI: 8945,06 – 69079,28	0,000

The presented data show that after treatment with alloferon and valacyclovir, the number of EBV DNA copies decreased in all patient groups.

In this work, we did not analyze the dynamics of clinical complaints in patients after therapy with alloferon and valacyclovir, since this was not the purpose of this work. Analysis of the dynamics of clinical complaints after alloferon was carried out in our previously published work [33]. A comparative analysis after therapy with valacyclovir will be carried out in the next work.

4. Discussion

Alloferon has a pronounced anti-inflammatory mechanism, regulates the production of pro-inflammatory cytokines. In their study, Zhang et al. demonstrated the anti-inflammatory properties of alloferon by using a mouse paw edema model caused by λ-carrageenan [34]. Administration of alloferon suppressed the expression of proinflammatory cytokines TNF-α and IL-1β ($P < 0.05$) in the inflamed paw tissue, i.e. alloferon inhibits the inflammatory response mediated by NK cells. Suppression of the expression of macrophage inflammatory protein MIP-1α and MIP-1β, monocyte chemoattractant protein 1 (MCP1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the λ-carrageenan-treated model, which are produced in response to various pro-inflammatory stimuli, provides new insights into the anti-inflammatory mechanisms of alloferon [34]. In the work of Qiao et al. on a mouse model of osteoporosis induced by estrogen deficiency, the anti-inflammatory mechanism of alloferon on the production of proinflammatory cytokines (IL-1β and IL-18) was shown [35]. In an experiment on mice, lipopolysaccharide induced endometritis. The authors of the study used immunofluorescence staining and western blotting to show that alloferon can modulate the production of inflammatory mediators. It has a strong anti-inflammatory effect, inhibiting the production of IL-1β and IL-18 and blocking the NLRP3/CASP1/IL-1β/IL-18 signaling cascade [36].

Our research shows that four weeks after finishing treatment with alloferon, the production of serum, spontaneous and induced IL-1β in the blood serum decreases. This happens in both groups of patients with EBV infection, regardless of their initial levels before treatment. In 2013, Kim Y. et al. published the results of a study on the effect of alloferon on the process of skin inflammation using the example of the human keratinocyte cell line HaCaT, caused by UV radiation [37]. Alloferon has been shown to reduce the production of proinflammatory cytokines such as IL-1α, IL-1β, IL-6 and IL-18 induced by UV radiation exposure at the mRNA and protein levels. Alloferon has been shown to reduce the production of proinflammatory cytokines such as IL-1α, IL-1β, IL-6 and IL-18 induced by UV radiation exposure at the mRNA and protein levels. MAPK activation plays an important role in the production of inflammatory cytokines upon UVB irradiation [38, 39]. The MAPK signaling pathway is involved in a variety of cellular processes including differentiation, development, proliferation and survival, as well as cell death, depending on the cell type and stimulus [40]. Kim A. L., et al. demonstrated that alloferon suppresses the activation of p38MAPK and NF-κB induced by UV irradiation [41]. p38 MAPK is a component of cytokine signaling pathways, and p38α is a key component of the IL-1 signaling pathway [42]. The data we obtained are completely consistent with the published results of other researchers, which confirms the pronounced anti-inflammatory effect of alloferon on the production of IL-1β in patients with chronic EBV infection.

In patients from the second group who had high levels of IL-1β production at the beginning, we found an increased amount of monocytes with the CD14+CD16+ phenotype compared to the first group. However, the number of cells was within the normal range. CD14+CD16+ cells are senescent cells that, when activated by antigen, acquire the functional and phenotypic characteristics of activated cells. They are capable of producing pro-inflammatory cytokines and stimulating the proliferation of Th2 lymphocytes [43]. In other words, these cells are dendritic cells [44]. An increase in the content of these cells in the peripheral blood induces a chronic inflammatory process characterized by the production of proinflammatory cytokines (IL-6, TNF-α) and a high level of IL-1β [45]. The analysis showed the presence of a regression model of the dependence of the level of induced IL-1β on the content of monocytes in the peripheral blood. This confirms the position that the inflammatory reaction developing against the background of EBV infection is accompanied by an increase in the content of CD14+CD16+ monocytes and the production of IL-1β. Yuka Torii et al. showed that EBV infection of monocytes increases caspase-1-dependent IL-1β production [46]. Monocytes lack the main EBV receptor CD21, but express CD35 and HLA-DR for

EBV [47]. In patients with EBV infection, linear and exponential regression of the dependence of IL-1β levels on the content of peripheral blood monocyte subpopulations was also revealed.

After therapy in patients of the control group with the drug valacyclovir, a reliable anti-inflammatory effect of the drug on the production of induced IL-1β was obtained in both subgroups of patients. The anti-inflammatory mechanism of valacyclovir has been described in the treatment of patients with Alzheimer's disease [48]. The researchers believed that taking valacyclovir, which targets the herpes simplex virus, could help reduce the chronic inflammation that leads to the development of the disease. As a result of antiviral therapy, patients showed a decrease in the production of TNF-α ($p<0.001$), IL-6 ($p<0.001$), IL-1β ($p<0.001$), IFN-γ ($p<0.001$). Thus, the anti-inflammatory activity of valacyclovir was demonstrated. The development of chronic neuroinflammation, the release of increased levels of proinflammatory cytokines and long-term use of antiviral drugs with a positive effect, in particular acyclovir and valacyclovir, have been described in other studies [49].

In an experiment with mice infected with the Herpes simplex virus type 1 (HSV1), scientists observed an inflammatory reaction in the central nervous system, which is known as encephalitis [50]. The authors investigated the effect of acyclovir on cytokine production in serum samples of infected mice. Intraperitoneal administration of acyclovir led to a significant decrease in IL-6 production and a decrease in monocyte infiltration. Satish K. et al studied the effect of valacyclovir on the production of proinflammatory cytokines in participants of Antarctic expeditions for prophylactic therapy during wintering in Antarctica [51]. All expedition participants were randomly assigned to two groups: a group receiving therapy (valaciclovir: 1 g/day), a group receiving placebo (oyster calcium: 500 mg/day). The research showed that valacyclovir significantly reduced the production of interleukin-6 (IL-6) by 2.5 times and interferon-gamma (IFN-γ) by 4 times.The level of IL-1β in the placebo group was 411.21±99.66 pg/ml, and in the valacyclovir group - 224.33±73.68 pg/ml. The authors of the study demonstrated not only a significant decrease in the number of viral DNA copies with prophylactic treatment with valacyclovir, but also a decrease in cytokine production, which is a consequence of a decrease in viral load as a result of therapy. L. Niu et al. in 2020 published the results of a retrospective study of the effectiveness of antiviral therapy with acyclovir in 96 children with viral encephalitis, assessing the effect of the drug on the production of IL-1 and IL-6 [52]. The authors of the work showed that acyclovir therapy causes a decrease in the production of proinflammatory cytokines. Thus, the results of our study completely coincide with the data published by other researchers using another pathology as an example.

In our study, we obtained a regression model of the relationship between the production of induced IL-1β and the number of EBV DNA copies in the general group of patients. That is, the number of EBV DNA copies can inhibit the production of induced IL-1β. In 1987, R.B. Acres et al showed that a line of EBV-infected peripheral blood B cells express the IL-1 beta gene [53]. Adachi H, et al, in an experiment on a line of EBV-infected human lung fibroblasts obtained by primary culture (CCD-32Lu), showed that EBV-infected fibroblasts are capable of producing basic fibroblast growth factor (bFGF) and IL-1β [54]. In 2024, Wenmin Zeng et al published the results of the effect of EBV on the inflammatory response of human gingival fibroblasts (HGF) in periodontitis. The authors studied the effect of EBV on the production of IL-1β, TNF-α and showed a significant increase in the level of these cytokines after EBV stimulation ($P < 0.01$) [55]. This work shows that EBV is involved in the activation of the TLR9 signaling pathway in HGF. That is, TLR9 is a potent regulator of cytokine production upon EBV induction in HGF, and the activation of the TLR9/MyD88/NF-κB signaling pathway plays an important role in EBV-dependent cytokine expression. These data are completely consistent with the data obtained in our study in patients with EBV infection and confirm the results described previously.

Our study showed that alloferon and valacyclovir have anti-inflammatory effects. However, when comparing these drugs, the following points are worth noting:

1. a course of alloferon therapy of 12 injections, a course of valacyclovir intake of at least 2 months;
2. alloferon does not have a toxic effect on internal organs;
3. good tolerability of alloferon;
4. high antiviral effect of alloferon [33];

Therefore, alloferon has an advantage for anti-inflammatory therapy in patients with EBV infection.

Summary

Our results showed that alloferon and valacyclovir have an anti-inflammatory mechanism on the production of proinflammatory cytokine IL-1β in patients with chronic Epstein-Barr virus infection. The production of IL-1β by cells is inhibited by the number of EBV DNA copies in the saliva sample.

Future research directions

It is advisable to conduct research to understand how alloferon affects the level of other types of peripheral blood mononuclear cells during treatment with the drug. Of interest is the study of the effect of alloferon therapy on the course of combined herpesvirus infection (Epstein-Barr virus and herpes virus type 6).

Data Sharing Policy. The statistical code, dataset used in support of the findings of this study are included within the article.

Financing. The study did not have sponsor’s support.

Conflict of interests. Authors declare the absence of conflict of interests.

Authors’ contribution.

conception and research design – Rakityanskaya I. A.;
material gathering and processing -- Rakityanskaya I. A., Ryabova T. S.;
data analysis and interpretation -- Rakityanskaya I. A., Ryabova T. S.;
lab research – Kalashnikova A.A.;
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research supervision – Rakityanskaya I.A.;
text writing and editing -- Rakityanskaya I. A., Ryabova T. S., Kalashnikova A.A.;
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Script further revision for important intellectual content -- Rakityanskaya I. A., Ryabova T. S., Kalashnikova A.A.
All the authors have made substantial contribution to this study and approved final script version.

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