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Research Article

Dynamics of Cytotoxic Activity of NK Cells During Therapy with Recombinant Interferon-Gamma in Patients with Chronic Epstein-Barr Virus Infection

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Abstract

Research Objective: NK cells are an important factor in the control of initial EBV infection because they eliminate infected B cells and enhance antigen-specific response of T cells by the release of immunomodulatory cytokines.

Materials and Methods: The research included 60 patients with chronic Epstein-Barr virus infection (CEBVI). The number of copies of EBV DNA in saliva before the start of therapy in patients was 298331.57 ± 8326.80 (95% CI: 166707.75 - 435596.23). The content of CD3−CD16+CD56+ cells was 10.95 ± 0.78 (95% CI: 9.53 - 12.58).

All patients were treated with Ingaron at a dose of 500,000 IU every other day IM. Initially, they received 10 injections of Ingaron followed by a 10 day break to assess the dynamics of clinical and laboratory parameters. Then the treatment was continued with 5 injections of recombinant interferon-gamma (Ingaron). In total, each patient received 15 injections or a total dose of 7,500,000 IU. EBV DNA quantity in the saliva samples was determined by the PCR method, also number of killer cells in blood; the killer cells cytotoxic activity was assessed via spontaneous and induced expression of marker of degranulation CD107a.

Results: The presence of NK cells in peripheral blood is significantly higher after administration of 10 injections of the drug and decreases after 15 injections. The introduction of recombinant IFN-γ at a total dose of 5,000,000 IU stimulates spontaneous and induced degranulation of NK cells in patients with CEBVI. The data show a significant decrease in the number of EBV DNA copies in saliva samples 10 days after a course of 10 injections (5,000,000 IU) of Ingaron; 21.66% of patients had a negative result of PCR test. After a full course of 15 injections of Ingaron, 31.66% of patients had a negative result.

Conclusions: 1. After administration of a total injection of 5,000,000 IU of ingaron (10 injections), there was a significant increase in the content of NK-cells. After administration of additional 2,500,000 IU of Ingaron (5 injections), i.e. when the course of 7,500,000 IU Ingaron was completed, the presence of NK cells decreased, but was still higher than before treatment. 2. Ingaron therapy stimulates of cytotoxic activity NK cells in patients with CEBVI. The maximum effect was obtained with the introduction of 5.000.000 IU of ingaron (10 injections) and it reduced after full course of 7,500,000 IU (15 injections) but did not return to initial values.

Keywords: *Chronic EBV Infection, Cytotoxic Activity NK Cells, Monotherapy, Interferon-Gamma, The Number of Copies of DNA*

Introduction

The Epstein-Barr virus (EBV) is a lymphotropic herpesvirus type 4 and the causative agent of infectious mononucleosis. EBV is widespread in all human populations and is tolerated by most people [1,2]. EBV infects B-lymphocytes and epithelial cells. Epithelial cells are the first to become infected, as the virus is transmitted in saliva from an infected person. Next, memory B cells are infected [3]. Infection occurs in two ways: 1. expression of latent genes leads to the transformation of B cells and the subsequent production of lymphoblastoid cell lines (LCLS); 2. expression of EBV lytic genes leads to the formation of infectious viral particles and lysis of the host cell [4]. In epithelial cells, EBV undergoes lytic replication, then establishes a lifelong latency in circulating memory B lymphocytes, and periodically reactivates from latency [5].

It has been shown that the infection of both cell types in the pharynx (in areas such as the tonsils) is critical to the persistence of the virus in the host. The virus is transmitted orally and is usually transmitted through close family contact during infancy or early childhood (under 5 years of age), when infection is almost always asymptomatic. However, in developed countries, primary infection is increasingly transmitted in the second or third decade of life [6]. EBV infects most people during their lifetime and persists for the rest of a person's life after the acute phase.

The life cycle of EBV is characteristic of a large DNA-enveloped virus consisting of phases of primary infection, latency, and lytic reactivation. During the lytic phase, the virus replicates and produces viral offspring. During the latent phase, the virus infects memory B cells, establishes lifelong infection, and avoids elimination by host cells [7]. Lytic replication increases the pool of latently infected cells. For cell envelope infection, the EBV genome encodes 9 different glycoproteins (GPS), 12 of which are only expressed during the productive cycle of lytic replication. During the latent period, only one GPS (BARF1 a decoy viral colony-stimulating factor 1 receptor (vCSF1R)) is expressed [3]. Infection of B cells and epithelial cells with EBV occurs via the CD21 receptor. When B cells are infected, the virus genome is preserved in the form of an episome.

A strong and sustained T-cell response is a major component of the immune control of EBV. Natural killers ((NK) [8] play a huge role in shaping the interaction of EBV with host cells. Natural killer cells (NK cells) are a unique subpopulation of cells that lacks antigen-specific receptors. NK cells have high cytotoxic activity and produce a large amount of interferon gamma (IFN-γ) when they interact with transformed or infected target cells [9]. NK cells (CD56+CD3-) account for approximately 5 - 20% of circulating lymphocytes in humans. They can be divided into two large subpopulations based on the expression of CD56 and CD16: CD56 dim CD16+ and CD56 bright CD16-. About 90% of NK cells are CD56 dim CD16+ ("mature" cytotoxic NK cells). They are present in the blood, bone marrow, spleen, and lungs with high cytotoxic potential. These cells produce high levels of interferon (IFN-γ), tumor necrosis factor (TNF-α), and cytolytic mediators (perforin and granzymes) [10,11]. The remaining 10% of NK cells are CD56 and CD16- ("immature" immunomodulatory NK cells). They are present in lymphoid tissues, tonsils and intestines. This subpopulation of NK cells is characterized by a decrease in cytotoxic activity and the ability to produce IFN-γ in the presence of interleukin (IL)-12 and IL-18 [11].

During EBV infection, NK cells expand in the peripheral blood by 1.7 times and increase cytotoxicity to destroy EBV-infected cells. A month later, the content of NK cells returns to its original levels. The expansion of early-differentiated NK cells lasts for at least six months; however, the cells in this period stop to proliferate and acquire CD57 marker of ageing [12,13]. A higher count of NK cells correlates with a lower EBV titer in peripheral blood, which suggests that the level of NK cell response depends on the clinical severity of the disease. It was recently demonstrated that induction of lytic replication in EBV-infected B cells leads to an increased destruction of NK cells. This may suggest that EBV-infected cells become a target for NK cells [14]. NK cells play an important role in the control of primary EBV infection by eliminating infected B cells and enhancing the antigen-specific T cell response through the release of immunomodulatory cytokines. However, 53% of patients with classic natural killer cell deficiency (CNKD) have severe herpesvirus infections, and some develop severe EBV disease. Lymphoproliferative disorders and persistent EBV viremia are observed in patients with a deficiency of the costimulatory CD27

molecule expressed on lymphocytes, including NK cells [8,15]. NK cells destroy EBV-infected cells undergoing lytic replication. Blazar B, Patarroyo M., *et al.* first reported this in 1980 [16]. Later, Pappworth I.Y., *et al.* demonstrated resistance of a latently infected B cell line to cell-mediated lysis by an NK cell line and primary polyclonal NK cells [17].

Induction of the EBV lytic phase of B cell lines by expression of a single EBV lytic gene (BZLF1) promotes sensitization of EBV-infected cells to NK cell cytotoxicity. BZLF1 is the master regulator of EBV lytic replication, playing a critical role in the life cycle of the virus. The ability of EBV to initiate a NK cell response to fight EBV viral infection is an important factor for a stable host-virus relationship that allows asymptomatic persistence of EBV. Luke R. Williams., *et al.* showed that, late in the lytic cycle, EBV-infected B cells become profoundly resistant to killing by NK cells [7]. During the late lytic phase, the BHRF1 gene is able to encode the viral homologue of the Bcl2 protein (vBcl2), and also contributes to the resistance of these EBV-infected B cell lines to NK-mediated killing due to increased resistance to apoptosis [7]. A subpopulation of NK cells (CD56bright CD16-NK cells) in the tonsils secrete higher levels of IFNγ than peripheral blood NK cells [18]. It has been shown that CD56bright NKG2A + CD56 tonsil NK cells are the most potent proliferation-limiting subpopulation of autologous EBV-infected tonsil B cells, in particular the germinal center or naïve B cell subpopulation [19]. The authors demonstrated that CD56bright NK2GA+ cells control EBV infection through IFN-γ secretion [19]. Taken together, these studies show the huge potential of tonsillar CD56bright NKG2A+ NK cells in restricting EBV-infected B cells.

In healthy people, the content of NK cells in the tonsils is from 0.2 to 1%, about 50 - 100 times lower than the percentage of NK cells in the peripheral blood or spleen. The peripheral blood mainly contains CD56dim, which express perforin and are cytolytic cells. NK cells in the tonsils are predominantly CD56bright, they usually do not express perforin, and have little cytotoxicity [20]. The low content of NK cells in the tonsils contributes to maintaining the number of EBV-infected memory B cells in the peripheral blood [17]. CD56 dim NKG2A+ NK cells predominantly identify autologous B cells with lytic EBV infection. This leads to the characteristic expansion of NK cells that has been identified in patients with acute MI [12].

It is assumed that NK cells have no significant control over the establishment of latency. Therefore, although the population of NK cells increases and is capable to kill target cells, no influence on the viral load during lytic or latent infection is observed. It was shown that NK cells play a crucial role in the control of herpes virus infections when the presence of viral antigens leads to the activation, proliferation, and accumulation of these cells in sites of infection [21]. Therefore, NK cells are an important factor in the control of initial EBV infection because they eliminate infected B cells and enhance antigen-specific response of T cells by the release of immunomodulatory cytokines.

Cytotoxicity of NK cells

NK cells infected with EBV deform, increase in size, and undergo transformation [22]. Every virus leads to a different phenotype of NK cells thus promoting persistence of the infection. Infected NK cells in turn increase the viral load. Inhibition or distortion of a small subpopulation of cells may significantly affect adaptive immune response to viral infection [23]. Natural cytotoxicity receptors (NCRs) including NKp30, NKp44, and NKp46 were the first identified group of receptors that bind viral glycoproteins. Every NCR can interact with several pathogen molecules encoded in the host and expressed on the cell membrane, secreted, released into the cell, or included into the extracellular matrix. It was shown that various isoforms of NCRs play a role as activating or inhibiting signals depending on their interaction with ligands [24]. As a result, the detection of infected cells activates NK cells and strongly induces their cytotoxicity [25]. Activated NK cells have three main strategies of eliminating virus-infected cells: 1) production of cytokines; 2) secretion of cytolytic granules; 3) death receptor-mediated cytolysis [26].

Degranulation is a hallmark of NK cell activation. Lytic granules, or secretory lysosomes packed with various proteins involved in cytotoxicity (e.g. perforins and granzymes), release their load on the surface of the target cell. Perforin molecules make pores in the membrane and increase its permeability. Granzymes, a family of serine proteases, disturb cell cycle, damage DNA, dissolve the nucleus of

the target cell, and induce its apoptosis by caspase-mediated signal paths. The internal surface of granules is covered with CD107a (lysosome-associated membrane protein 1), a highly glycosylated protein that appears on the cell surface because lysosomes merge with the cell membrane. Degranulation leads to the expression of CD107a on the cell surface and to the depletion of intracellular perforins. After degranulation, CD107a is exposed on the surface of cytotoxic lymphocytes and protects the membrane from perforin-mediated damage [27,28]. All dormant NK cells can express CD107a on their surface and mediate cytotoxicity after they receive a signal for degranulation. The two stages of cytotoxicity of NK cells, i.e. polarization and degranulation of cytolytic granules, are controlled by different signals from different receptors. Neither polarization nor degranulation is sufficient for effective lysis of target cells, but coagulation of CD16 and LFA-1 leads to a strong cytotoxicity of dormant NK cells. It was shown that one activated NK cell is able to kill four target cells within 16 hours [29]. The depletion of perforin in granules may affect the capacity of NK cells to destroy virus-infected cells [30,31]. Therefore, NK cells can kill such cells before «depletion», which in part may be connected with the depletion of cytolytic granules [32].

Antiviral functions of IFN-γ

After they find a way into the body of the potential host, viruses affect mucus membranes and move with the blood flow. They also spread with mobile cell and along neural paths and replicate in living host cells [33]. Infection starts when the virus attaches to the cell membrane using a receptor and/or non-specifically via surface receptor molecules. In order to replicate, viruses must deliver their genome to the host cell and use its cellular mechanisms. The effective infection occurs through the following sequence of events: a). binding to receptors on the cell membrane; b). internalization; c). release from endocytosis vesicles; d). delivery of genome to the nucleus.

IFN-γ has a direct antiviral action on infected cells, and also activates local dendritic cells, macrophages, and NK cells, modulates differentiation and maturing of T cells and B cells, and promotes inflammation and antiviral functions [34]. Suppression of any stage of the life cycle of virus can suppress the replication of its genome during infection. IFN-γ is a powerful antiviral cytokine that disrupts the life cycle of virus in stimulated cells on various stages. There are several mechanisms of its action:

1. It inhibits virus infiltration on extracellular and intracellular stage by controlling expression and/or distribution of respective receptors;
2. It inhibits replication by disrupting the replication niche of the virus;
3. It disrupts gene expression by preventing translation;
4. It prevents the assembly of the nucleocapsid by affecting its stability;
5. It disrupts the release of virus by breaking the disulfide bridge, a significant part of cellular interactions;
6. It suppresses the main regulator of viral transcription and changes reactivation of viruses;
7. It can inhibit the infiltration of invasive viruses on the stage of their transition from endosome to cytoplasm [34].

Some well-known antiviral functions of IFN-γ lack specific antiviral mechanism.

In the Russian Federation, the only IFN-γ drug registered under the trade name Ingaron, developed by OOO NPP "PHARMACLON" by microbiological synthesis in recombinant strain *E. coli* and purified by column chromatography. Ingaron consists of 144 amino acid residues, devoid of the first three of them - Cys-Tyr-Cys, replaced by Met.

Aim of the Study

The aim of this study was to investigate the influence of recombinant IFN-γ (Ingaron) on the cytotoxic activity of killer cells in patients with chronic Epstein-Barr virus infection (CEBVI) after the treatment.

Materials and Methods

Patients

The study group included 60 patients with CEBVI (39 women and 21 men; mean age 34.64 ± 1.21 years). The duration of CEBVI from first complaints to laboratory confirmation and diagnosis was 2.85 ± 0.56 years. 43 patients (71.66%) had frequent exacerbations of antibiotic-resistant chronic tonsillitis in childhood, and 15 patients (25%) had a history of acute infectious mononucleosis. All patients had differential diagnosis of CEBVI versus other viral infections (human immunodeficiency virus, viral hepatitis, cytomegalovirus infection), toxoplasmosis, helminth infestations, and autoimmune diseases associated with EBV infection. The diagnosis was confirmed on a previous stage by laboratory investigation and expert examination, and the patients were referred for immunological treatment. Anti-VCA IgM+, anti-VCA IgG+ and anti-EBNA1 IgG were detected in serum and Viral DNA was detected in saliva samples using real-time polymerase chain reaction (PCR).

Those patients, who received antiviral and immunomodulatory therapy within the last 6 months, were not included into the study.

This clinical study was performed in accordance with World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects (2013); the protocol to the Convention of the Council of Europe on Human Rights and Biomedicine (1999); and Articles 20, 22, 23 of the Russian Federal Law no. 323-FZ On fundamental healthcare principles in the Russian Federation (21 November 2011 as revised on 26 May 2021). The protocol was approved by the ethical committee of OOO Tsentr dializa Sankt-Peterburg, Fresenius Medical Care. All participants signed a voluntary informed consent. Patients included into the study had no other diagnosed infections, chronical diseases, or changed immune status that could affect the results.

Treatment schedule

All patients received therapy with intramuscular recombinant IFN-γ (Ingaron) at a dose of 500,000 IU every other day. The course consisted of 15 injections. In the first phase, patients received 10 injections (5,000,000 IU) of Ingaron at a single dose of 500,000 IU followed by a 10 day break to assess the dynamics of clinical and laboratory parameters. After that, the therapy was resumed and patients received 5 injections (2,500,000 IU) of Ingaron. 10 days after the last injection, examination was repeated. In total, every patient received 15 injections (7,500,000 IU) of Ingaron.

All patients tolerated the drug fairly well. After the first 3 to 5 injections, 14 patients (23.33%) had a fever (37.3 - 37.5°C), myalgia, chills, sore throat, and increased post-nasal drip. This was considered an exacerbation of CEBVI in association with the drug. After 7th and 8th injection, these complaints fully disappeared.

Methods of examination. Viral DNA was detected in saliva samples using real-time polymerase chain reaction (PCR) with fluorescence hybridization

Ampli Sens EBV/CMV/HHV6-screen-FL kits by the Central Research Institute of Epidemiology (Russia) were used. The unit of measurement used to estimate the viral load during DNA extraction from saliva is the number of copies of EBV DNA per ml of sample. Accord-

ing to the instructions, this indicator is calculated using the formula: Number of DNA copies = CDNA x 100, where CDNA is the number of copies of the viral DNA in the sample. The analytical sensitivity of the test system is 400 copies/ml.

Cytotoxic activity of killer cells was evaluated based on spontaneous and induced expression of CD107a (LAMP, lysosomal-associated membrane protein) on the cell membrane of lymphocytes, which is a sign of degranulation of lysosomes. CD107a was assessed after co-culture of peripheral blood mononuclear cell (PBMC) with target cells (K562, chronic human erythromyelosis). K562 cells express a range of ligands (MICA, MICB, ULBP2, ULBP4) for NKG2D receptor of cytotoxic lymphocytes. The interaction between NKG2D and the ligands leads to degranulation of lysosomes in NK cells, TNK cells, and lymphokine-activated CD8+ T cells, and to the expression of CD107a on their membranes. Therefore, the test reveals the ability of killer cells to participate in NKG2D-dependent cytolysis of target cells. Blood was collected in a vacutainer with heparin lithium as an anticoagulant. Sample preparation included separation of mononuclear cells suspension from peripheral blood using density gradient with subsequent washing, co-culture of PBMC and K562 in 10:1 ratio in a CO_2 incubator for 20 hours with anti-CD107a-AlexaFluor 647 monoclonal antibodies (Bio Legend); and staining with anti-CD3-FITC/CD(CD16+56)-PE and anti-CD45PC5 monoclonal antibodies (Beckman Coulter). To assess spontaneous cytotoxic activity, a respective volume of RPMI medium (Biolot) was added to PBMC suspension instead of K562. The samples were analyzed using Navios flow cytometer (Beckman Coulter) up to 1,000 events in a minimum subpopulation of NK or TNK cells. The population of lymphocytes was defined as CD45+brightSSdim. The relative number of cells with CD107a expression (CD107a+) was assessed in subpopulations of NK, TNK, and T lymphocytes. The stimulation index was calculated as a ratio of induced expression to spontaneous expression of CD107a.

To assess the relative number of NK cells multicolor flow cytometry was applied during the study of lymphocyte subpopulations in peripheral blood collected from the ulnar vena in vacutainers with EDTA. The samples were prepared according to the manufacturer's protocol. The following monoclonal antibodies were used: anti-HLADR-FITC, anti-CD4-PE, anti-CD3-ECD, anti-CD56-PC5.5, anti-CD25-PC7, anti-CD8-APC, anti-CD19-APC-AF700, and anti-CD45-APC-AF750. VersaLyse was chosen for the lysis of red blood cells. The samples were analyzed using Navios flow cytometer and respective reagents (Beckman Coulter) up to 5,000 events from the CD45+brightSSdim lymphocytic region. NK cells were defined as CD3−CD56+ CD45+brightSSdim events. The absolute number of NK cells was calculated from the results of clinical blood analysis.

Statistical analysis

IBM SPSS Statistics ver. 26 software package (Armonk, NY: IBM Corp.) was used for statistical analysis of the data. Group results were presented as the mean (M) ± standard deviation (SD). Statistical comparison between groups of patients was performed using non-parametric Mann-Whitney U test. Differences in continuous variables were assessed using independent samples Student's t-test and were considered statistically significant if $p \leq 0.05$.

Results

The effectiveness of treatment with recombinant IFN-γ (Ingaron)

In all patients (n = 60), EBV infection was confirmed by PCR reaction in saliva samples. The study of DNA PCR was carried out 10 days after the administration of 10 injections of ingaron (total 5,000,000 IU). After that, patients received 5 more injections of Ingaron (2,500,000 IU), and the number of copies of EBV DNA in saliva samples was assessed by PCR again. The results are shown in table 1.

The data show a significant decrease in the number of EBV DNA copies in saliva samples 10 days after a course of 10 injections (5,000,000 IU) of Ingaron; 21.66% of patients had a negative result of PCR test. After a full course of 15 injections (7,500,000 IU) of Ingaron, 31.66% of patients had a negative result of PCR test of saliva samples (Figure 1). This means that the effectiveness of antiviral therapy confirmed by negative PCR was significantly higher after 15 injections than after 10 injections ($p = 0.001$).

Group of patients	Copies/ml before treatment	Copies/ml 10 days after 10 injections	Copies/ml 10 days after 15 injections	P
	1	2	3	
Ingaron 500,000 IU, IM every other day	298331.57 ± 8326.80 (n = 60), 95% CI: 166707.75-435596.23	177369.51 ± 3994.40 (n = 47) 95% CI: 85699.01-326572.72 13 patients (21.66%) had 0.00 copies	8593.92 ± 3248.46 (n = 41) 95% CI: 2422.26-13232.15 19 patients (31.66%) had 0.00 copies	P1,2 = 0.0001 P1,3 = 0.0001 P2,3 = 0.001

Table 1: *The dynamics of the number of copies of EBV DNA after treatment with Ingaron in patients with CEBVI.*

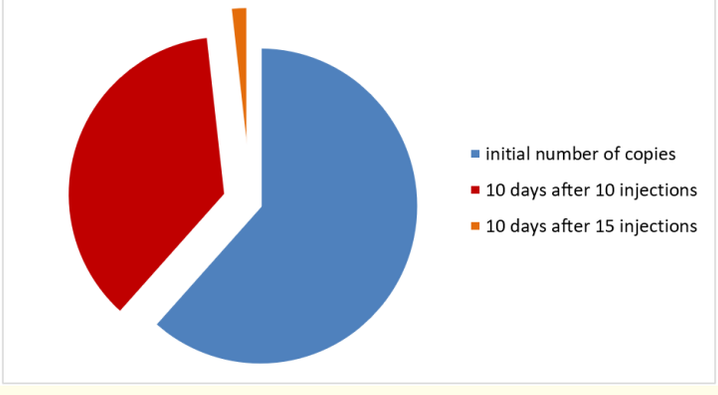

Figure 1: *The dynamics of EBV DNA in saliva samples before and after treatment with Ingaron in patients with CEBVI.*

Presence of NK cells in peripheral blood

The presence of NK cells in peripheral blood was assessed before treatment, after 10 injections, and after 15 injections of Ingaron. The results are shown in table 2 and figure 2.

Subpopulations of mononuclear cells in blood, %	Before treatment with Ingaron	10 days after 10 injections	10 days after 15 injections	p
	1	2	3	
CD3−CD16+CD56+	10.95 ± 0.78 95% CI: 9.53-12.58	15.37 ± 0.96 95% CI: 13.59-17.28	12.33 ± 0.76 95% CI: 10.93-13.72	P1,2 = 0.001 P1,3 = 0.006 P2,3 = 0.001
CD3+CD16+CD56+	6.97 ± 0.63 95% CI: 5.81-8.16	9.46 ± 0.65 95% CI: 7.60-12.25	5.89 ± 0.68 95% CI: 4.52-7.18	P1,2 = 0.031 P1,3 = 0.328 P2,3 = 0.001
CD3+CD16+CD56−	2.97 ± 0,33 95% CI: 2.34-3.66	4.74 ± 0.56 95% CI: 3.64-6.20	3.89 ± 0.31 95% CI: 3.30-4.50	P1,2 = 0.001 P1,3 = 0.031 P2,3 = 0.04

Table 2: *The content of NK cells (%) in blood before and after the treatment with Ingaron in patients with CEBVI.*

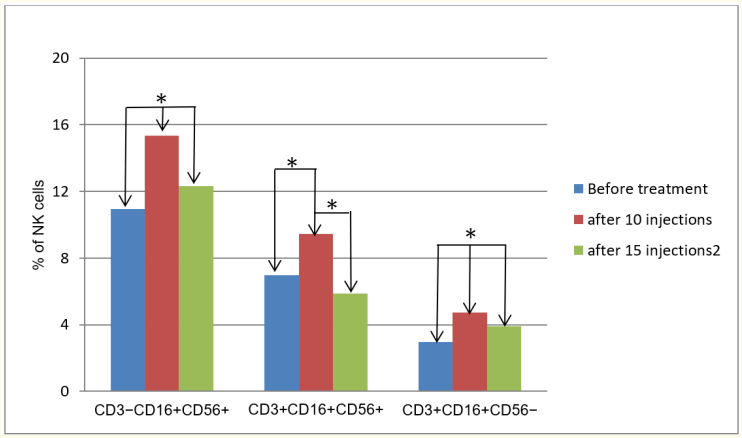

Figure 2: *The content of NK cells (%) in blood before and after the the treatment with Ingaron in patients with CEBVI (* - p<0,05).*

The data show that the presence of NK cells in peripheral blood is significantly higher after administration of 10 injections of the drug and decreases after 15 injections, but generally still exceeds the level before treatment.

Dynamics of cytotoxic activity of NK cells

Next, the dynamics of cytotoxic activity of NK cells before treatment and 10 days after 10 injections of Ingaron was assessed (Table 2). The expression of CD107a on NK cells 10 days after 10 injections of Ingaron significantly increased and exceeded referent values. This means that the introduction of recombinant IFN-γ at a total dose of 5,000,000 IU stimulates spontaneous and induced degranulation of NK cells and stimulation index in patients with CEBVI. After a full course of treatment (7,500,000 IU of recombinant IFN-γ), the expression of CD107a on NK cells reduced but was still higher than before treatment and exceeded referent values. Therefore, the maximum activity of NK cells in peripheral blood in patients with CEBVI was observed 10 days after administration of a total dose of 5,000,000 IU Ingaron (Table 3).

Expression of CD107a	Before treatment	10 days after the first stage of treatment (10 injections)	Reference values	p
Expression of degranulation marker CD107a on CD3−CD16+CD56+ cells				
Spontaneous	2.94 ± 0.35 95% CI: 2.31-3.72	5.22 ± 0.40 95% CI: 3.47-5.09	0.9-3.3	p = 0.001
Induced	19.20 ± 1.12 95% CI: 16.98-21.39	22.06 ± 1.09 95% CI: 19.22-3.50	11.0-24.0	p = 0.003
Stimulation index	11.05 ± 0.91 95% CI: 10.20-16.47	15.22 ± 1.05 95% CI: 12.17-16.45	5.5-17.0	p = 0.001
Expression of degranulation marker CD107a on CD3+CD16+CD56+ cells				
Spontaneous	1.46 ± 0.15 95% CI: 1.16-1.78	2.60 ± 0.25 95% CI: 1.52-2.50	0.4-1.6	p = 0.004
Induced	2.50 ± 0.26 95% CI: 2.02-3.02	5.01 ± 1.47 95% CI: 2.69-8.45	0.5-3.0	p = 0. 001

Stimulation index	2.27 ± 0.30 95% CI: 1.75-2.95	3.59 ± 0.58 95% CI: 2.54-4.80	1.0-2.5	p = 0.024
Expression of degranulation marker CD107a on CD3+CD16+CD56− cells				
Spontaneous	0.31 ± 0.02 95% CI: 0.25-0.37	0.71 ± 0.13 95% CI: 0.47-1.02	0.1-0.4	p = 0.009
Induced	0.34 ± 0.03 95% CI: 0.26-0.42	1.23 ± 0.18 95% CI: 0.88-1.59	0.1-0.4	p= 0.0001
Stimulation index	1.14 ± 0.06 95% CI: 1.02-1.28	1.62 ± 0.15 95% CI: 1.35-1.94	≤ 1.0	p = 0.002

Table 3: *The dynamics of the expression degranulation marker CD107a on NK cells, before treatment and 10 days after 10 injections of Ingaron in patients with CEBVI.*

Next, the dynamics of cytotoxic activity of NK cells 10 days after 15 injections of Ingaron was analyzed (Table 4).

Expression of CD107a	Before treatment	10 days after the second stage of treatment (15 injections)	Reference values	P
Expression of degranulation marker CD107a on CD3− CD16+ CD56+ cells				
Spontaneous	2.94 ± 0.35 95% CI: 2.31-3.72	3.99 ± 0.41 95% CI: 3.26 - 4.86	0.9 - 3.3	p = 0.056
Induced	19.20 ± 1.12 95% CI: 16.98-21.39	21.08 ± 1.01 95% CI: 19.05-23.04	11.0 - 24.0	p = 0.02
Stimulation index	11.05 ± 0.91 95% CI: 10.20-16.47	13.08 ± 0.99 95% CI: 11.12-15.07	5.5-17.0	p=0.0001
Expression of degranulation marker CD107a 0n CD3+CD16+CD56+ cells				
Spontaneous	1.46 ± 0.15 95% CI: 1.16-1.78	2.25 ± 0.26 95% CI: 1.74-2.78	0.4-1.6	p = 0.005
Induced	2.50 ± 0.26 95% CI: 2.02-3.02	3.39 ± 0.31 95% CI: 2.99-5.11	0.5-3.0	p = 0.04
Stimulation index	2.27 ± 0.30 95% CI: 1.75-2.95	3.62 ± 0.63 95% CI:2.82-4.00	1.0-2.5	p = 0.01
Expression of degranulation marker CD107a on CD3+CD16+CD56- cells				
Spontaneous	0.31 ± 0.02 95% CI: 0.25-0.37	0.51 ± 0.09 95% CI:0.34-0.72	0.1-0.4	p = 0.062
Induced	0.34 ± 0.03 95% CI: 0.26-0.42	1.08 ± 0.17 95% CI: 0.73-1.42	0.1-0.4	p=0.0001
Stimulation index	1.14 ± 0.06 95% CI: 1.02-1.28	1.59 ± 0.13 95% CI: 1.35-1.88	≤ 1.0	p = 0.004

Table 4: *The dynamics of the expression degranulation marker CD107a on NK cells, before treatment and 10 days after 15 injections of Ingaron in patients with CEBVI.*

The data from table 4 are shown on figure 3.

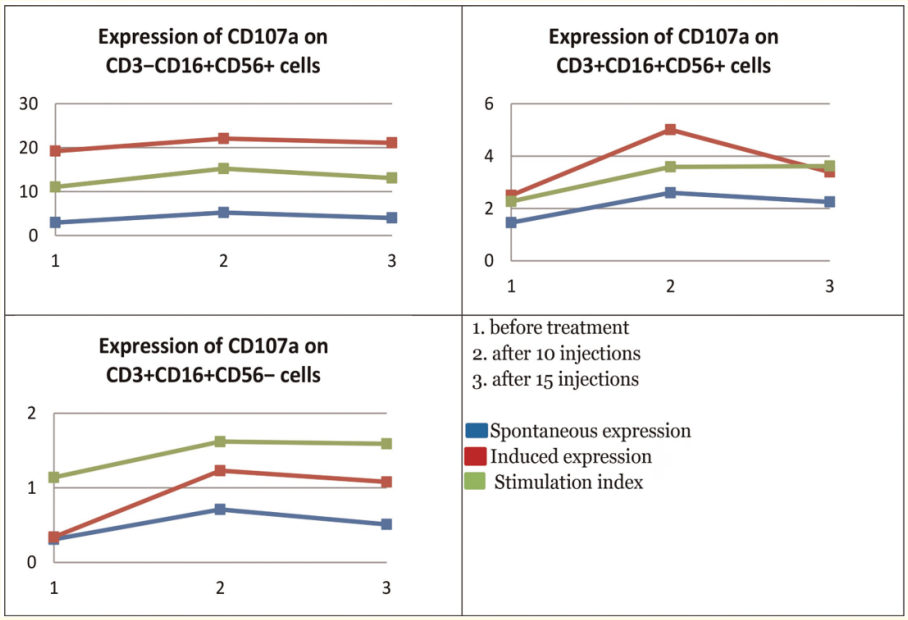

Figure 3: *The dynamics of the expression of CD107a marker of degranulation of cytotoxic granules by NK cells before and after 10 and 15 injections of Ingaron in patients with CEBVI.*

The dynamics of the content of NK cells and cytotoxic activity visually resemble the sign "bell" or "arch" (∩) of varying severity. This direction of the obtained results indicates the development of a hyporeactive state of cells against the background of a longer administration of ingaron (15 injections). The hyporeactive state of NK cells is a consequence of a decrease in the number of EBV DNA copies, which in turn is accompanied by a positive dynamics of clinical complaints after a full course of therapy (7.500.000 IU).

Discussion

NK cells play an important role in combating viral infections. In 1986, Kure S., *et al.* demonstrated that none of the rhIFNs lack pronounced inhibiting effect on EBNA expression in hidden EBV-infected Raji and Daudi cells. These results suggest that rhIFN act mostly on the early stage of EBV infection [35]. It was demonstrated in an experimental setting that pre-treatment of Vero cells with either IFN-β or IFN-γ inhibits HSV-1 replication by less than 20-fold. Co-treatment with IFN-β and IFN-γ inhibits HSV-1 replication about 1,000 times [36]. The authors proposed that a high level of inhibition after the introduction of exogenous IFN-γ was a result of a synergic interaction with endogenous IFN-α/IFN-β produced locally in response to HSV-1 infection. A study of influence of purified recombinant interferons of all three classes on EBV-induced proliferation of B cells and immunoglobulin secretion showed that IFN-γ reduces B cell proliferation and immunoglobulin production if added 3 - 4 days after infection, and that IFN-α and IFN-β effectively influence cell proliferation only within 24 hours. The authors showed that the antiviral effect of IFN-γ on EBV-infected cells is 7 - 10 times stronger than that of IFN-α and IFN-β [37].

Our study demonstrated a significant decrease in the number of copies of EBV DNA in saliva samples 10 days after the administration of 5,000,000 IU of Ingaron, and the results of PCR test were negative in 21.66% of patients. After a full course of treatment with 7,500,000

IU Ingaron, 31.66% of patients had negative results of PCR test of saliva samples. This means that the full course of Ingaron is significantly more effective ($p = 0.001$).

The state of the latent or replicative lytic cycle stimulates the expansion of NK cells, which depends on the number of lytically infected cells. One reason for this may be the loss of inhibitory signals supplied by HLA class I molecules to target cells. Lytic replication is responsible for the expansion of early differentiated subpopulations of NK cells and is probably targeted by NK cells [38]. Therefore, EBV infection leads to the expansion of NK cells in blood; this precedes a peak of T cell response and induces a prolonged differentiation of mainly CD16+NKG2A+KIR−NK cells, which is an evidence of an early differentiation phenotype [39]. Williams Hilary., *et al*. demonstrated that an increased viral load negatively correlates with the share of NK cells in peripheral blood (Pearson correlation coefficient was - 0.87, $p < 0.001$) [40]. A higher EBV load reflects a high percentage of cells with latent infection in circulating subpopulation of B cells. It was shown that the replication level of EBV in saliva samples has no association with the severity of symptoms of infectious mononucleosis [41]. We also found no correlation between the total number of NK cells in the blood and the viral load in saliva samples, which coincides with previously published data in the work of Rachel J. Abbott., *et al.* It is not possible to detect EBV DNA in blood samples at this stage due to the chronic course of infection, when EBV replication occurs in the oropharynx and the virus is released into saliva for a long time [42].

In our study, the expression of CD107a degranulation marker on NK cells 10 days after administration of 5,000,000 IU Ingaron significantly increased and excessed reference values. This means that the introduction of recombinant IFN-γ at a total dose of 5,000,000 IU stimulates spontaneous and induced degranulation of NK cells in patients with CEBVI. After the full course of treatment with 7,500,000 IU of recombinant IFN-γ, the expression of CD107a on NK cells decreased but was still higher than before the treatment and exceeded reference values. The maximum activity of NK cells in peripheral blood of patients with CEBVI was achieved 10 days after administration of a total dose of 5,000,000 IU Ingaron. Therefore, the results of the analysis of NK cells degranulation correlate with standard results on cytotoxicity as shown in studies by Alter G., *et al*. [43,44]. The expression of CD107a can therefore be a sensitive marker of cytotoxic activity of NK cells. The maximum expansion of NK cells in peripheral blood of patients with CEBVI was observed after administration of a total dose of 5,000,000 IU Ingaron. After additional 5 injections (2,500,000 IU) Ingaron, i.e. after a full course of 7,500,000 IU Ingaron, the content of NK-cells decreased, but did not reach the initial level. The dynamics content and cytotoxic activity of NK cells visually resemble the sign “bell” or “arch” (∩) of a different curvature.

In 1985, Talmadge JE., *et al*. were the first to demonstrate the bell-like curve of the dependency of NK cells presence on the dose of recombinant IFN-γ *in vitro* and *in vivo* [45]. They experimented on mice and showed that the activity of NK cells sharply increases 24 hours after administration of recombinant IFN-γ and reaches a peak 48 hours after administration. The drug was several times more effective to increase cytotoxicity mediated by NK cells compared to IFN-α; its repeated administration led to a decrease in NK cells activity, and a hyporesponsive state developed. Pre-clinical and clinical studies of recombinant IFN-γ also showed a bell-like dependency on the dose when NK cells were induced by multiple or high doses of the drug [46,47]. In this case, the hyporesponsiveness of NK cells occurred when normal cells stimulated NK cells but the inhibiting signals from HLA Class I molecules were absent, or when excessive stimulation were stronger than inhibiting signals. Constant engagement of activating receptors and the lack of inhibiting receptors led to the hyporesponsiveness of NK cells [48]. Experiments on mice showed that the constant interaction of the activating Ly49H receptor with NK cells leads to the development of hyporeactivity of NK cells due to changes in the downstream signaling pathways from the receptor to the adapter molecule [49]. Constant interaction of Ly49H receptor with its ligand *in vivo* results in a weak response of Ly49H+ NK cells to further stimulation from other receptors whereas Ly49H− NK cells remain unaffected. Hyporesponsiveness of NK cells correlates with the suppression of the activity of Ly49H receptor on the cell membrane. When effective inhibiting signals are absent, NK cells experience sustained activation and become hyporeactive, which is known as the “disarming” model [49]. However, the most important mechanisms that lead to hyporesponsiveness of NK cells need further investigation.

Based on the previously published results of studies on the mechanism of development of NK cell hyporeactivity and our data, it becomes obvious that long-term administration of recombinant interferon-γ in patients with chronic EBV infection leads to the develop-

ment of a decrease in the function of NK cells. In our study, the development of a hyporeactive state of NK cells against the background of a longer administration of ingaron (15 injections) is accompanied by a decrease in the number of copies of EBV DNA in saliva samples and a more pronounced positive dynamics of clinical complaints in patients after a full course of therapy (7.500.000 IU).

Conclusions

1. Ingaron is a recombinant human INF-γ preparation. It has a pronounced antiviral effect, which is expressed in a significant decrease in the number of EBV DNA copies in patients with CEBVI.

2. After administration of a total injection of 5,000,000 IU of ingaron (10 injections), there was a significant increase in the content of NK-cells, which indicates the effect of ingaron on the development of the maximum expansion of NK-cells in patients with CEBVI.

   After administration of additional 2,500,000 IU of Ingaron (5 injections), i.e. when the course of 7,500,000 IU Ingaron was completed, the presence of NK cells decreased, but was still higher than before treatment.

3. Ingaron therapy stimulates spontaneous and induced degranulation of NK cells, that is, cytotoxic activity in patients with CEBVI. The maximum effect was obtained with the introduction of 5.000.000 IU of ingaron (10 injections) and it reduced after full course of 7,500,000 IU (15 injections) but did not return to initial values.

Considering our results (the effect of the recommended interferon-gamma (Ingaron) on the expansion of NK cells and the activation of cytotoxic activity of cells in CEBVI patients), it seems interesting to further study the mechanisms of cytotoxicity activation by studying the secretion of perforin and granzyme under the influence of Ingaron. It is also of interest to study the effect of recombinant interferon-gamma on changes in the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in patients with CHEBVI after therapy.

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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.; statistical processing of data - Rakityanskaya I. A.;script composition-- RakityanskayaI.A.,RyabovaT.S.;editing - Ryabova T. S.Kalashnikova A.A.; research supervision - Rakityanskaya I.A.;text writing and editing -- Rakityanskaya I. A., Ryabova T. S., Kalashnikova A.A.; responsibility for integrity of all article's parts - Rakityanskaya I.A.; 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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