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Chapter

Recombinant Interferon Gamma: Influence on the Cytotoxic Activity of NK Cells in Patients with Chronic Epstein-Barr Virus Infection

Irina A. Rakityanskaya, Tatiana S. Ryabova and Anastasiya A. Kalashnikova

Abstract

NK cells play an important role in combating viral infections. In this study, we examined the effect of therapy with recombinant interferon gamma (Ingaron) on cytotoxic activity of NK cells. Sixty patients with chronic Epstein-Barr virus infection (CEBVI) were examined. 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 five injections of Ingaron. In total, each patient received 15 injections or a total dose of 7,500,000 IU. The administration of recombinant interferon gamma at a total dose of 5,000,000 IU stimulated spontaneous and induced degranulation of NK cells in patients with CEBVI. After a full course of 7,500,000 IU of recombinant interferon gamma, CD107a expression on NK cells decreased but remained higher than before the onset of therapy and exceeded reference values. Thus, the maximum activity of NK cells in the peripheral blood of patients with CEBVI was reached 10 days after the administration of Ingaron at a total dose of 5,000,000 IU.

Keywords: NK cells, cytotoxic activity, chronic Epstein-Barr virus infection, recombinant interferon gamma, therapy

1. Introduction

1.1 Epstein-Barr virus

The Epstein-Barr virus (EBV) is a lymphotropic herpesvirus type 4 and the causative agent of infectious mononucleosis [1, 2]. The virus was first discovered and isolated in cells from African Burkitt’s lymphoma by Epstein M.A., Barr Y.M., and
Achong B.G. in 1964 and later it was found that EBV is widespread throughout the world [3]. The first identified variants of EBV were type 1 (type A) and type 2 (type B). Type 1 (B95-8, GD1, and Akata) is the main type of EBV prevalent worldwide and type 2 (AG876 and P3HR-1) is more common in Sub-Saharan Africa [4]. EBV variants have different replicative properties and a person can become superinfected with two or more strains.

EBV infects most people during their lifetime and, after the acute phase, persists until the end of a person’s life. The life cycle of EBV is characteristic of a virus with a large DNA envelope, consisting of phases of primary infection, latency, and lytic reactivation. The EBV genome encodes nine different glycoproteins (GPS) for envelope entry. Currently, 13 GPS have been identified, 12 of which are only expressed during the productive cycle of lytic replication. One of which (BARF1, a decoy viral colony-stimulating factor 1 receptor (vCSF1R)) can also be expressed during the latency period [5]. The tropism of newly released EBV virions is determined by the GPS envelope, which appears to differ depending on the host cell [6]. EBV infects B cells via the CD21 receptor, epithelial cells, and, less commonly, T or NK cells. Infection of B-lymphocytes leads to the preservation of the EBV genome as an episome.

The virus undergoes lytic replication in epithelial cells and establishes a lifelong latency in circulating memory B lymphocytes, periodically reactivating from latency [7]. Epithelial cells are the first to become infected, as EBV is transmitted to recipients via saliva. B cells become infected when EBV is released from the oropharyngeal epithelial cells [6, 8]. Lytic replication increases the pool of latently infected cells. EBV virions released from epithelial cells prefer B cells and EBV virions released from B cells prefer epithelial cells due to the composition of the GPS envelope [9]. EBV reactivation (lytic phase) under conditions of psychological stress leads to a weakening of cellular immunity and can stimulate EBV reactivation and replication by weakening the cellular immune system’s control over viral latency. Chronic EBV reactivation is an important mechanism in the pathogenesis of many oncological and autoimmune diseases [10]. During the lytic phase, the full set of virus genes is expressed and a progeny virus is produced. Virions produced during lytic replication in epithelial cells replenish the viral reservoir in an infected individual and ensure the transmission of the virus in the population. During the latency period, the virus expresses only a limited number of genes necessary to maintain the viral genome (in the form of an episome in the nucleus) and evade the host’s immune system [8].

1.2 Natural killer cells

Natural killer cells (NK cells) are a unique subpopulation of cells that lack 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 [11]. The recognition process of target cells consists of the signals they receive from activating and inhibitory receptors encoded by the germline. As a result of these interactions, the identification or death of target cells occurs.

In the absence of inhibiting signal, continuous stimulation of activation receptors deactivates NK cells and reduces their activity. When target cells transform or become infected, the expression of HLA Class I on their surface may cease. Therefore, multiple NK cell receptors along with the presence of activated cytokines and cells that adapt and express various receptors in NK cell compartment promote responsiveness of these innate cytotoxic lymphocytes [12].
The activity of NK cells is also regulated by four additional mechanisms:

1. repertoire of NK cells;
2. activation by cytokines or priming of NK cells;
3. adaptive or memory-like differentiation of NK cells; and
4. licensing of NK cells.

There are 30,000 subpopulations of NK cells that differ in respect of inhibiting and activating receptor expression.

During Epstein-Barr virus (EBV) infection, NK cell expansion occurs in peripheral blood, and the cytotoxicity of NK cells to EBV-infected cells increases. The expansion of early differentiated NK cells lasts for at least 6 months [13]; however, the cells in this period stop to proliferate and acquire CD57 marker of aging [14]. 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. 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 [15]. 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.

1.3 Antiviral functions of IFN-γ

Currently, there are specific antiviral drugs, but there is no single approach to the treatment of chronic EBV infection. The antiherpetic drug must specifically inhibit the replication of the virus. The moment the virus evades the host's immune response, it is a potential target for chemotherapeutic effects. The higher the selectivity of the drug, the narrower the spectrum of its antiviral activity, since the drugs affect only the stages of virus replication. Drugs approved for the treatment of herpes simplex virus 1 (HSV-1) and 2 (HSV-2), varicella-zoster virus (VZV), and human cytomegalovirus (HCMV) are nucleoside (i.e., acyclovir (ACV), penciclovir (PCV), ganciclovir (GCV), and its oral prodrugs; valacyclovir (VACV), famciclovir (FAM), and valganciclovir (VGCV), respectively), nucleotide (i.e., cidofovir (CDV)), and pyrophosphate (i.e., foscavir (foscarnet sodium), PFA) [16, 17]. None of these drugs have received FDA (Food and Drug Administration) or EMA (European Medicines Agency) approval for the treatment of EBV infections [8, 18].

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 [19]. 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 stages 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; and

7. It can inhibit the infiltration of invasive viruses on the stage of their transition from endosome to cytoplasm [19].

Some well-known antiviral functions of IFN-γ lack specific antiviral mechanism. For instance, IFN-γ strongly induces indoleamine-2,3-dioxygenase (IDO) and nitric oxide synthase (NOS). The depletion of tryptophan and the production of nitric oxide (NO) due to the expression of IDO and NOS have pronounced antiviral effects, but their molecular details generally remain unclear. IFN-γ can also manifest non-cytolytic activity against some viruses. However, specific targets and effector proteins of IFN-γ-dependent antiviral response are largely unknown [20]. Further studies are needed to clarify the antiviral mechanisms of IFN-γ, especially considering its strong immunomodulatory action.

In Russia, the only registered IFN-γ drug is Ingaron manufactured by OOO NPP FARMKLON. It is obtained by the microbiological synthesis in recombinant Escherichia coli strain and purified by column chromatography. The molecule consists of 144 amino acid residues; the first three residues (Cys-Tyr-Cys) are replaced with Met.

The objective of the present research is to study the recombinant IFN-γ (Ingaron) action on dynamics of content of EBV DNA in the saliva sample, the killer cells content post-therapy, and changes of cytotoxic activity of the killer cells, and assess the influence of cytotoxic activity of the natural killers on the clinical complaint development and progression of illness in patients with CEBVI after the therapy completion.

2. 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 was from first complaints to laboratory confirmation and the diagnosis was 2.85 ± 0.56 years. Forty-three 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 a 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 the immunological treatment. Those
patients, who received antiviral and immunomodulatory therapy within the last 6 months, were not included in the study.

CEBVI characterizes with a prolonged treatment and frequent recurrences with clinical and laboratory signs of viral activity (mononucleosis-like symptoms) that are described in detail in the literature [21]. Patients suffer from low-grade fever (37.1—37.3 °C), weakness, unmotivated tiredness, excessive sweat (especially at night), constant discomfort and/or pain in throat, lymphadenitis, swelling of the nasal mucosa with postnasal mucus drip, and stomatitis. Some patients have cough, skin eruptions, arthralgia, and muscle pain in body and limbs. Manifestation of conjunctivitis and otitis is possible. Neurological disorders such as headache, impaired memory and sleep, impaired concentration, irritability, tearfulness, and depressive tendencies may occur. Internal organs may increase in size (hepatomegaly and splenomegaly evidenced by ultrasound investigation) and a heavy feeling under the right ribs may be present. Some patients complain about frequent cold-related diseases and concurrent herpes virus infections. Many of these patients have a history of prolonged stress and psychoemotional and physical overload that exacerbates their condition.

This clinical study was performed in accordance with the 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 (November 21, 2011 as revised on May 26, 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.

**Clinical methods** included taking of history, data on previous treatment, and concurrent diseases. The clinical condition of patients was assessed traditionally with consideration of objective data and complaints at the time of examination registered using a three-point scale (0—no symptoms, 1—mild symptoms, 2—moderate symptoms, and 3—severe symptoms).

**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. In total, 500,000 units are the standard daily dose of the drug, which is recommended by the manufacturer. After that, the therapy was resumed and patients received five injections (2,500,000 IU) of Ingaron. Ten days after the last injection, the examination was repeated. In total, every patient received 15 injections (7,500,000 IU) of Ingaron (see Figure 1).

![Figure 1. Treatment regimen.](http://dx.doi.org/10.5772/intechopen.108207)
All patients tolerated the drug fairly well. After the first 3–5 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 the seventh and eighth injections, these complaints fully disappeared.

### 3. Methods of examination using real-time polymerase chain reaction (PCR) with fluorescence hybridization

Viral DNA was detected in saliva samples using real-time polymerase chain reaction (PCR) with fluorescence hybridization, AmpliSens 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. According 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 the 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, and ULBP4) for NKG2D receptor of cytotoxic lymphocytes. The interaction between NKG2D and the ligands leads to the 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 CO₂ incubator for 20 hours with anti-CD107a-AlexaFluor 647 monoclonal antibodies (BioLegend), and staining with anti-CD3-FITC/CD(CD16+56)-PE and anti-CD45PC5 monoclonal antibodies (Beckman Coulter). To assess the spontaneous cytotoxic activity, a respective volume of RPMI medium (Biolot) was added to PBMC suspension instead of K562. The samples were analyzed using a 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 nonparametric Mann—Whitney U test. Differences in continuous variables were assessed using independent samples Student’s t-test and were considered statistically significant if \( p \leq 0.05 \). Parametric (Pearson correlation) and nonparametric (Spearman’s rank, Kendall’s tau) methods were also applied. To check the independence of observations, linear regression analysis with the coefficient of determination (\( R^2 \)), Durbin—Watson statistic, and analysis of variance (ANOVA) were applied. Fisher’s exact test (F) was calculated to check the statistical significance of the model. A standard \( \beta \) coefficient with 95% confidence intervals was calculated. The threshold significance of differences in this study was 0.05.

4. Results

4.1 The effectiveness of treatment with recombinant IFN-\( \gamma \) (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 five 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 2). 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).

4.2 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 3.

<table>
<thead>
<tr>
<th>Group of patients</th>
<th>Copies/ml before treatment</th>
<th>Copies/ml 10 days after 10 injections</th>
<th>Copies/ml 10 days after 15 injections</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingaron 500,000 IU, IM every other day</td>
<td>298331.57 ± 8326.80 (n = 60), 95% CI: 166707.75—435596.23</td>
<td>177369.51 ± 3994.40 (n = 47)</td>
<td>8593.92 ± 3248.46 (n = 41)</td>
<td>P1,2 = 0.0001 P1,3 = 0.0001 P2,3 = 0.001</td>
</tr>
</tbody>
</table>

Table 1. The dynamics of the number of copies of EBV DNA after treatment with Ingaron in patients with CEBVI.
Table 2.
The content of NK cells (%) in blood before and after the treatment with Ingaron in patients with CEBVI.

<table>
<thead>
<tr>
<th>Subpopulations of mononuclear cells in blood, %</th>
<th>Before treatment with Ingaron</th>
<th>10 days after 10 injections</th>
<th>10 days after 15 injections</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3−CD16+CD56+</td>
<td>10.95 ± 0.78</td>
<td>15.37 ± 0.96</td>
<td>12.33 ± 0.76</td>
<td>P1,2 = 0.001</td>
</tr>
<tr>
<td>95% CI: 9.53—12.58</td>
<td>95% CI: 13.59—17.28</td>
<td>95% CI: 10.93—13.72</td>
<td></td>
<td>P1,3 = 0.006</td>
</tr>
<tr>
<td>CD3+CD16+CD56+</td>
<td>6.97 ± 0.63</td>
<td>9.46 ± 0.65</td>
<td>5.89 ± 0.68</td>
<td>P1,2 = 0.031</td>
</tr>
<tr>
<td>95% CI: 5.81—8.16</td>
<td>95% CI: 7.60—12.25</td>
<td>95% CI: 4.52—7.18</td>
<td></td>
<td>P1,3 = 0.328</td>
</tr>
<tr>
<td>CD3+CD16+CD56−</td>
<td>2.97 ± 0.33</td>
<td>4.74 ± 0.56</td>
<td>3.89 ± 0.31</td>
<td>P1,2 = 0.001</td>
</tr>
<tr>
<td>95% CI: 2.34—3.66</td>
<td>95% CI: 3.64—6.20</td>
<td>95% CI: 3.30—4.50</td>
<td></td>
<td>P1,3 = 0.031</td>
</tr>
</tbody>
</table>

*p* values indicate statistical significance.

Figure 2.
The dynamics of EBV DNA in saliva samples before and after treatment with Ingaron in patients with CEBVI.

Figure 3.
Dynamics of the content of NK-cells (%) in the blood before and after treatment with Ingaron in patients with CHEBVI.
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.

4.3 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).

<table>
<thead>
<tr>
<th>Expression of CD107a</th>
<th>Before treatment</th>
<th>10 days after the first stage of treatment (10 injections)</th>
<th>Reference values</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expression of degranulation marker CD107a on CD3⁺CD16⁺CD56⁺ cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Spontaneous</strong></td>
<td>2.94 ± 0.35</td>
<td>5.22 ± 0.40</td>
<td>0.9—3.3</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>95% CI: 2.31—3.72</td>
<td>95% CI: 3.47—5.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Induced</strong></td>
<td>19.20 ± 1.12</td>
<td>22.06 ± 1.09</td>
<td>11.0—24.0</td>
<td>p = 0.003</td>
</tr>
<tr>
<td>95% CI: 16.98—21.39</td>
<td>95% CI: 19.22—23.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stimulation index</strong></td>
<td>11.05 ± 0.91</td>
<td>15.22 ± 1.05</td>
<td>5.5—17.0</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>95% CI: 10.20—16.47</td>
<td>95% CI: 12.17—16.45</td>
<td></td>
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<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Expression of degranulation marker CD107a on CD3⁺CD16⁺CD56⁻ cells</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spontaneous</strong></td>
<td>1.46 ± 0.35</td>
<td>2.60 ± 0.25</td>
<td>0.4—1.6</td>
<td>p = 0.004</td>
</tr>
<tr>
<td>95% CI: 1.16—1.78</td>
<td>95% CI: 1.52—2.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Induced</strong></td>
<td>2.50 ± 0.26</td>
<td>5.01 ± 1.47</td>
<td>0.5—3.0</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>95% CI: 2.02—3.02</td>
<td>95% CI: 2.69—8.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stimulation index</strong></td>
<td>2.27 ± 0.30</td>
<td>3.59 ± 0.58</td>
<td>1.0—2.5</td>
<td>p = 0.024</td>
</tr>
<tr>
<td>95% CI: 1.75—2.95</td>
<td>95% CI: 2.54—4.80</td>
<td></td>
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<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Expression of degranulation marker CD107a on CD3⁺CD16⁺CD56⁻ cells</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spontaneous</strong></td>
<td>0.31 ± 0.02</td>
<td>0.71 ± 0.13</td>
<td>0.1—0.4</td>
<td>p = 0.009</td>
</tr>
<tr>
<td>95% CI: 0.25—0.37</td>
<td>95% CI: 0.47—1.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Induced</strong></td>
<td>0.34 ± 0.03</td>
<td>1.23 ± 0.18</td>
<td>0.1—0.4</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>95% CI: 0.26—0.42</td>
<td>95% CI: 0.88—1.59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stimulation index</strong></td>
<td>1.14 ± 0.06</td>
<td>1.62 ± 0.15</td>
<td>≤ 1.0</td>
<td>p = 0.002</td>
</tr>
<tr>
<td>95% CI: 1.02—1.28</td>
<td>95% CI: 1.35—1.94</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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).

The data from Table 4 are shown in Figure 4.

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).

### 4.4 Dynamics of clinical complaints

The next stage of the work was an analysis of the frequency of the main clinical complaints in patients before treatment and after 10 and 15 injections of Ingaron. Table 5 and Figure 5 show the dynamics of clinical complaints during therapy.

<table>
<thead>
<tr>
<th>Expression of CD107a</th>
<th>Before treatment</th>
<th>10 days after the second stage of treatment (15 injections)</th>
<th>Reference values</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expression of degranulation marker CD107a on CD3−CD16+CD56+ cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>2.94 ± 0.35</td>
<td>3.99 ± 0.41</td>
<td>0.9—3.3</td>
<td>p = 0.056</td>
</tr>
<tr>
<td>95% CI: 2.31—3.72</td>
<td>95% CI: 3.26—4.86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induced</td>
<td>19.20 ± 1.12</td>
<td>21.08 ± 1.01</td>
<td>11.0—24.0</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>95% CI: 16.94—21.39</td>
<td>95% CI: 19.05—23.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation index</td>
<td>11.05 ± 0.91</td>
<td>13.08 ± 0.99</td>
<td>5.5—17.0</td>
<td>p = 0.0001</td>
</tr>
<tr>
<td>95% CI: 10.20—16.47</td>
<td>95% CI: 11.12—15.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Expression of degranulation marker CD107a on CD3+CD16+CD56+ cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>1.46 ± 0.15</td>
<td>2.25 ± 0.26</td>
<td>0.4—1.6</td>
<td>p = 0.005</td>
</tr>
<tr>
<td>95% CI: 1.16—1.78</td>
<td>95% CI: 1.74—2.78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induced</td>
<td>2.50 ± 0.26</td>
<td>3.39 ± 0.31</td>
<td>0.5—3.0</td>
<td>p = 0.04</td>
</tr>
<tr>
<td>95% CI: 2.02—3.02</td>
<td>95% CI: 2.99—3.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation index</td>
<td>2.27 ± 0.30</td>
<td>3.62 ± 0.63</td>
<td>1.0—2.5</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>95% CI: 1.75—2.95</td>
<td>95% CI: 2.82—4.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Expression of degranulation marker CD107a on CD3+CD16+CD56- cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>0.31 ± 0.02</td>
<td>0.51 ± 0.09</td>
<td>0.1—0.4</td>
<td>p = 0.062</td>
</tr>
<tr>
<td>95% CI: 0.25—0.37</td>
<td>95% CI: 0.34—0.72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induced</td>
<td>0.34 ± 0.03</td>
<td>1.08 ± 0.17</td>
<td>0.1—0.4</td>
<td>p = 0.0001</td>
</tr>
<tr>
<td>95% CI: 0.26—0.42</td>
<td>95% CI: 0.73—1.42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation index</td>
<td>1.14 ± 0.06</td>
<td>1.59 ± 0.13</td>
<td>≤ 1.0</td>
<td>p = 0.004</td>
</tr>
<tr>
<td>95% CI: 1.02—1.28</td>
<td>95% CI: 1.35—1.88</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 show that after the introduction of 10 injections of ingaron, there is a significant decrease in the frequency of subfebrile temperature, sore throat, weakness, and manifestations of stomatitis. After the introduction of 15 injections of ingaron, the dynamics of clinical complaints are more evident: a decrease in the frequency of subfebrile temperature, sore throat, weakness, chills, stomatitis, and swelling of the nasal mucosa with postnasal mucus drip.

Table 5.
The frequency (%) of main clinical complaints before treatment and after 10 and 15 injections of recombinant IFN-γ in patients with CEBVI.

<table>
<thead>
<tr>
<th>Frequency of clinical complaints, %</th>
<th>Before treatment (n = 60)</th>
<th>10 days after 10 injections</th>
<th>10 days after 15 injections</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subfebrile temperature</td>
<td>83.33</td>
<td>45.00</td>
<td>36.66</td>
<td>P1,2 = 0.004, P1,3 = 0.001, P2,3 = 0.003</td>
</tr>
<tr>
<td>Lymphadenitis</td>
<td>50.00</td>
<td>46.66</td>
<td>41.66</td>
<td>P1,2 = 0.08, P1,3 = 0.052, P2,3 = 0.07</td>
</tr>
<tr>
<td>Sore throat</td>
<td>85.00</td>
<td>58.33</td>
<td>40.00</td>
<td>P1,2 = 0.001, P1,3 = 0.001, P2,3 = 0.001</td>
</tr>
<tr>
<td>Weakness</td>
<td>75.00</td>
<td>58.33</td>
<td>46.66</td>
<td>P1,2 = 0.01, P1,3 = 0.001, P2,3 = 0.05</td>
</tr>
<tr>
<td>Chills</td>
<td>71.66</td>
<td>68.33</td>
<td>50.00</td>
<td>P1,2 = 0.074, P1,3 = 0.001, P2,3 = 0.001</td>
</tr>
<tr>
<td>Excessive sweat</td>
<td>88.33</td>
<td>80.00</td>
<td>46.66</td>
<td>P1,2 = 0.052, P1,3 = 0.0001, P2,3 = 0.001</td>
</tr>
<tr>
<td>Swelling of the nasal mucosa with postnasal mucus drip</td>
<td>35</td>
<td>30.00</td>
<td>18.33</td>
<td>P1,2 = 0.08, P1,3 = 0.05, P2,3 = 0.07</td>
</tr>
<tr>
<td>Stomatitis</td>
<td>31.66</td>
<td>21.66</td>
<td>16.66</td>
<td>P1,2 = 0.05, P1,3 = 0.001, P2,3 = 0.07</td>
</tr>
<tr>
<td>Irritability and tearfulness</td>
<td>60.00</td>
<td>56.66</td>
<td>53.33</td>
<td>P1,2 = 0.058, P1,3 = 0.054, P2,3 = 0.072</td>
</tr>
<tr>
<td>Headaches, dizziness</td>
<td>35.00</td>
<td>31.66</td>
<td>30.00</td>
<td>P1,2 = 0.068, P1,3 = 0.052, P2,3 = 0.07</td>
</tr>
<tr>
<td>Impaired concentration and memory</td>
<td>40.00</td>
<td>38.33</td>
<td>33.33</td>
<td>P1,2 = 0.082, P1,3 = 0.056, P2,3 = 0.058</td>
</tr>
<tr>
<td>Disturbed sleep</td>
<td>41.66</td>
<td>38.33</td>
<td>35.00</td>
<td>P1,2 = 0.058, P1,3 = 0.070, P2,3 = 0.072</td>
</tr>
</tbody>
</table>
Figure 4.
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.

Figure 5.
The frequency (%) of main clinical complaints before treatment and after 10 and 15 injections of recombinant IFN-γ in patients with CEBVI.
4.5 Prognostic value of the presence of CD3−CD16+CD56+ cells in peripheral blood

To reveal the prognostic value of NK cells, linear regression analysis was performed with coefficients of determination (R²) calculated using Durbin—Watson statistic, and also the analysis of variance (ANOVA), Fischer exact test (F), and standard beta coefficient (β) with 95% confidence interval. The results of the criterion F and the coefficient β, indicating the significance of the obtained regression models, are presented below:

1. The content of CD3−CD16+CD56+ cells in the blood before treatment contributes to the appearance of sore throat (F = 4.186; p = 0.009; β = 0.457; CI: 0.796; 4.237; p = 0.022).

2. The content of CD3−CD16+CD56+ cells in the blood before treatment influences the development and progression of disturbed sleep (F = 7.762; p = 0.007; β = 0.324; CI: 0.773; 4.683; p = 0.007).

3. The content of CD3−CD16+CD56+ cells in the blood before treatment influences the development and progression of stomatitis (F = 3.256; p = 0.045; β = 0.211; CI: -0.187; 2.873; p = 0.043).

4. The content of CD3−CD16+CD56+ cells before treatment influences the development and progression of irritability and tearfulness (F = 4.420; p = 0.039; β = 0.251; CI: 0.091; 3.519; p = 0.030).

The results of linear regression show that the presence of CD3−CD16+CD56+ subpopulation of cells in blood before treatment is a predictor of the development and progression of clinical complaints in patients with CEBVI.

5. Resume

NK cells play a critical role in fighting EBV infection. NK cells are cytotoxic to EBV-transformed cells during the acute phase and limit the EBV viral load [22]. The mechanism of action of NK cells against EBV is not well understood. NK cell cytotoxicity is strongly activated by EBV-induced ligands on infected B cells. Activated NK cells use three main strategies to kill virus-infected cells:

a. production of cytokines;

b. secretion of cytolytic granules; and

c. death receptor-mediated cytolysis.

NK cells can prevent EBV entry into B cells and prevent B cell transformation via IFN-γ [23]. Human peripheral blood NK cells recognize EBV-replicating B cells by suppressing MHC class I surface molecules on infected cells [24].

The human NK cell compartment has up to 30,000 different subpopulations. Human herpesviruses promote the expansion of distinct subpopulations of NK cells, which then persist at an increased frequency for several months after infection.
During this time, they stop proliferating and acquire the aging marker CD57. Uncontrolled EBV infection develops with a decrease in NK cell compartments [25]. The hallmark of NK cell activation is degranulation, that is, the release of the contents of lytic granules. The granules consist of secretory lysosomes containing a dense core, various proteins, and take part in cytotoxic functions (e.g., perforin, granzymes) on the surface of the target cell. The inner surface of the granules is covered with CD107a (lysosome-associated membrane protein 1), a highly glycosylated protein that appears on the cell surface due to the fusion of lysosomes with the plasma membrane. Degranulation leads to the expression of CD107a on the cell surface and depletion of intracellular perforin. After degranulation, CD107a is exposed on the surface of the cytotoxic lymphocyte, protecting the membrane from perforin-mediated damage [26]. Resting NK cells, upon receiving signals for degranulation, are able to express surface CD107a and mediate cytotoxicity. Polarization and degranulation of cytolytic granules are two steps in NK cell cytotoxicity that are controlled by separate signals from different receptors. Neither polarization nor degranulation is sufficient for the efficient lysis of target cells. The ability of NK cells to kill virus-infected cells occurs before the “depletion” of NK cells, which is probably due to the depletion of cytolytic granules. The results of the NK cell degranulation analysis have been shown to correlate with standard cytotoxicity results. That is, CD107a expression may be a sensitive marker for determining cytotoxic activity [27].

In our study, the expression of CD107a degranulation marker on NK cells 10 days after the administration of 5,000,000 IU Ingaron significantly increased and exceeded 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 the peripheral blood of patients with CEBVI was achieved 10 days after the 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. [27]. The expression of CD107a can therefore be a sensitive marker of cytotoxic activity of NK cells. The maximum expansion of NK cells in the peripheral blood of patients with CEBVI was observed after the administration of a total dose of 5,000,000 IU Ingaron, after additional five injections (2,500,000 IU) Ingaron, that is, 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, J. E. 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 [28]. They experimented on mice and showed that the activity of NK cells sharply increases 24 hours after the 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 with IFN-α; its repeated administration led to a decrease in NK cells activity, and a hyporesponsive state developed. Preclinical 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 [29]. This systemic hyporeactive state occurs not only in the spleen and peripheral blood, but also in NK cells isolated from the lungs and liver. 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 was stronger than inhibiting signals. Constant engagement of activating receptors and the lack of inhibiting receptors led to the hyporesponsiveness of NK cells.

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. The 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 [30]. However, the most important mechanisms that lead to the 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 development 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).

The study of the inhibitory effect of pure recombinant human (rh) IFN-α and IFN-γ on EBV infection began in the late 80s and early 90s of the twentieth century. In 1986, Shigeo Kure 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 [31]. It was demonstrated in an experimental setting that pretreatment 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 [32, 33]. 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 the 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 [34, 35]. 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). A strong and significant decrease in clinical complaints of patients was achieved after the full course of treatment.

6. 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 (five injections), that is, 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.

4. The content of CD3+CD16+CD56+ cells in the blood before treatment is a predictor of the development and progression of clinical complaints in patients with CEBVI.

5. The effectiveness of therapy in patients with CVEI, both in relation to clinical complaints and the number of copies of EBV DNA in saliva, is determined by the duration of administration of recombinant human INF-γ (Ingaron). At least 15 injections of 500,000 IU Ingaron every other day are required.

7. Future research directions

It is necessary to carry out further investigation of how Ingaron affects dynamics of content of other subpopulations of lymphocytes of peripheral blood in the course of treatment by the medication. Also seems to be interesting to study production of the anti-inflammatory cytokines (IL-1β, IL-6, and TNF-β) in the course of the Ingaron treatment.

Based on preliminary results of this study, we suppose that Ingaron possesses manifest anti-viral action and is one of the activators of immune response. The medication can be used as a combination therapy for chronic Epstein-Barr infection, which will save working population and reduce burden on the healthcare system.

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—Rakityanskaya I. A., Ryabova T. 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 a substantial contribution to this study and approved the final script version.
Conflict of interests

The authors declare the absence of conflict of interests.

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.

Author details

Irina A. Rakityanskaya1*, Tatiana S. Ryabova1,2 and Anastasija A. Kalashnikova3

1 Department of Allergology, Immunology and Clinical Transfusiology, Municipal Outpatient Hospital, Saint Petersburg, Russia

2 S.M. Kirov Military Medical Academy, Saint Petersburg, Russia

3 A.M. Nikiforov Russian Center of Emergency and Radiation Medicine, EMERCOM of Russia, Saint Petersburg, Russia

*Address all correspondence to: tat-akyla@inbox.ru

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