Investigation of IL-2 and IFN- γ to EBV Peptides in Stimulated Whole Blood among Multiple Sclerosis Patients and Healthy Individuals

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Keywords

Epstein-Barr virus · Multiple sclerosis · Interleukin-2 · Interferon- γ

Abstract

Introduction: Epstein-Barr virus (EBV), a double-stranded DNA virus, has 2 phases of lytic and latent infection in host cells. After infecting B lymphocytes, EBV becomes persistent in these cells. In healthy individuals, T lymphocytes play a key role in killing EBV-infected B cells. Statistical studies have shown that symptomatic EBV infection increases the risk of MS. **Methods:** This study intended to measure the immune system's response against the different components of EBV, focusing particularly on T lymphocytes' reaction. Consequently, the mRNA level of IL-2 and IFN- γ , liable for impressing autoimmune diseases and as indicators of T-cell function, was compared in EBNA1- and BRLF1-treated whole blood (WB) cultures of 10 healthy individuals and 10 MS patients using real-time RT-PCR. **Results:** The analysis of the results demonstrated a significant increased level of IL-2 in MS patients than healthy subjects after exposure to both peptides. Also, the mRNA level of IFN- γ increased in MS patients in EBNA1-treated WB culture. **Conclusion:** According to the study's results, EBV peptides can reactivate immune cells, es-

pecially T lymphocytes, and may indirectly induce inflammation and develop MS; however, it seems that long-time exposure to these peptides has reducing effect on T-cell function and faces the control of infected B lymphocytes with difficulties.

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Introduction

Human herpesvirus 4, better known as Epstein-Barr virus (EBV), belongs to the family of Herpesviridae [1]. With a circular double-stranded DNA genome, EBV is similar to human chromosome in many molecular facets [2]. EBV infects at least 90% of all human adults throughout the world [3]. The primary cellular target of EBV is mostly B lymphocytes and epithelial cells, but natural killer (NK) cells and T lymphocytes can also be infected. Ensuing primary infection, EBV sets up a latent infection in memory B cells, while expressing latent genes and stays hidden from the host immune system [4–6]. Latent EBV genomes express 2 latent membrane proteins and 5 EBV-encoded nuclear antigens (EBNA); among them, EBNA1 is the only antigen expressed in all 3 forms of latent infection [7].

EBV infection can lead to a wide variety of diseases from infectious mononucleosis to EBV-associated malignancies; in addition, many studies have shown a relationship between EBV and autoimmune diseases including multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system, in which myelinated axons are damaged [3, 8]. In healthy individuals, EBV-specific immune responses, especially CD8⁺ T cells, control EBV infection by eliminating infected B cells; however, in MS patients, they show progressive exhaustion [9]. Exhausted T cells initially obtain effector function but gently become silenced because of successive T-cell receptor stimulation from persistent antigen [10]. T-cell exhaustion is determined by downregulation of effector function and reduction in type I cytokine secretion such as IL-2 and IFN- γ and upregulation of inhibitory molecules [11].

Based on the alteration of EBV-specific T-cell function in MS patients, the aim of the current study was to establish a comparison in immune cell response, after their exposure to EBV peptides. To provide a culture that is physiologically similar to in vivo condition, we used whole blood (WB), a suitable substitute for peripheral blood mononuclear cells (PBMCs), because purification of PBMC using Ficoll results in selective loss of gamma delta T lymphocytes and other important components impacting cellular response and IFN- γ levels [12, 13]. Furthermore, WB assay is an easy and fast method which sustains the integrity of cellular components of blood and allows different elements to have essential interactions equivalent to in vivo situations [14, 15].

Materials and Methods

Subjects and Sample Processing

This study used the WB sample from 10 MS patients who were diagnosed with relapsing-remitting multiple sclerosis and also 10 healthy donors (Table 1). The study protocol was approved by the Medical Ethics Committee of Tarbiat Modares University (IR.TMU.REC.1396.704).

Whole venous blood was drawn into an EDTA tube and diluted 1:1 in sterile RPMI 1640 culture medium (BIO-IDEA, Iran). Each blood sample was divided into 3 study groups; 2 of them were treated with 1 μ g/mL final concentration of antigen peptide EBV BRLF1 HLA-A*2402 (DYCNVLNKEF) and antigen peptide EBV EBNA1 HLA-B*3501 (HPVGEADYFEY) (JPT Peptide Technologies, Germany), and the last one remained untreated. After 72 h of incubation, supernatant was collected, and total RNA was extracted using RNSol H reagent (ROJETechnologies, Iran) according to manufacturer's instruction and stored at -70°C . cDNAs were synthesized from extracted RNAs using a cDNA synthesis kit (Biofact, South Korea) pursuant to the kit's protocol and stored at -20°C .

Table 1. Characteristics of MS patients and healthy control subjects

	MS patients*	Healthy control subjects
Age, mean (range)	34 (29–44)	33.6 (25–51)
Gender		
Female	7	6
Male	3	4
White blood cell count, mean	6,323	6,873

MS, multiple sclerosis. * All MS patients were diagnosed with relapsing-remitting MS and also took interferon beta as medication.

MTT Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay was carried out in accordance with MTT Assay kit (BIO-IDEA) guidelines with a final concentration of 1 μ g/mL of each peptide, at 24, 48, 72, and 96 h of cell incubation, and absorbance changes were measured at 570 and 640 nm using an ELISA plate reader.

Real-Time RT-PCR and Statistical Analysis

Primers were designed using DNASTAR Lasergene software and synthesized by Metabion (Germany). Real-time reactions were made using RealQ plus 2X Master Mix Green high ROX (Ampliqon, Denmark) according to the manufacturer's instruction. For fluorescent reporter dye, the real-time master mix had SYBR Green. Collected relative gene expression data were analyzed by the $2^{-\Delta\Delta\text{CT}}$ (Livak) method, and statistical analyses were performed using GraphPad Prism 8.

Results

MTT Test Results

MTT test showed no cytotoxicity of peptides in different hours of cell culture incubation. In Figure 1, cell viability percentage of different groups is illustrated in comparison with WB culture of 24 h. In addition, the viability percentage of treated cells did not significantly differ from the control (untreated) group in each incubation time.

Increase of IL-2 mRNA Levels in MS Patients

IL-2 mRNA levels of all groups were evaluated by real-time RT-PCR. Results from calculating fold change amount using real-time RT-PCR data were analyzed and charted in Figure 2 by the one-way ANOVA test. The comparison of 4 study group's fold change indicated a remarkable increase in IL-2 mRNA after treating with EBV peptides in MS patients ($p < 0.05$). In both BRLF1-

Fig. 1. Cell viability percentage at 24, 48, 72, and 96 h of WB, treated with BRLF1 and EBNA1 peptides. Cell viability shown in every group was in comparison with the first 24 h and indicated no remarkable difference as well as in comparison with control (untreated) groups. WB, whole blood.

Fig. 2. Tukey's multiple comparisons of IL-2 mRNA levels in healthy individuals and MS patients based on fold change. IL-2 mRNA amount of MS patients was higher in comparison with healthy subjects in both BRLF1- and EBNA1-treated groups (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). MS, multiple sclerosis.

Fig. 3. Tukey's multiple comparisons of IFN- γ mRNA levels in MS patients and healthy individuals based on fold change. IFN- γ mRNA levels showed a significant increase in EBNA1-treated WB of MS patients ($p < 0.05^*$). MS, multiple sclerosis; WB, whole blood.

and EBNA1-treated WB, MS patients had higher IL-2 mRNA amounts, compared to healthy individuals.

Increase of IFN- γ Levels after Treating with EBNA1 Peptide in MS Patients

According to analyzed RT-PCR results by one-way ANOVA (shown in Fig. 3), a notable multiplication of IFN- γ mRNA levels ($p < 0.05$) was noticed after treating WB with the EBNA1 peptide in MS patients while there

was not a considerable increase in the BRLF1-treated group when compared with healthy subjects.

IL-2 mRNA Had Higher Levels in MS EBNA1-Treated Group than IFN- γ

Two-way ANOVA test was used to compare IL-2 and IFN- γ with each other. As shown in Figure 4, in EBNA1-treated WB in MS, IL-2 mRNA demonstrated a higher amount than IFN- γ . However, a significant difference be-

Fig. 4. Sidak's multiple comparisons of IL-2 and IFN- γ mRNA levels in study groups. IL-2 showed a higher level of mRNA than IFN- γ in EBNA1-treated WB (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001). WB, whole blood.

tween IL-2 and IFN- γ in an identical group was only confined to EBNA1-treated WB and could not be observed in any other group.

Discussion

EBV causes several lymphatic diseases [16]. It usually begins its lytic phase in the tonsils epithelium and expresses latent proteins in naive B cells [17]. In primary infection, infected B cells are under intense control of T lymphocytes; however, in latency, EBV becomes hidden from the immune system and remains persistent in resting memory B cells until it becomes activated again [18]. While it is expected to observe higher viral load in reactivated EBV, a study in Qatar surprisingly has reported no significant contrast in different stages of infection. This result implicates the steady activation of EBV regardless of the infection phase [19].

MS is characterized by pervasive myelin attacks due to infiltration of immune cells. Although it was presumed for decades that CD4⁺ T cells solely played a key role in MS development, further studies have revealed the substantial impact of CD8⁺ T cells in creating the disease [20, 21]. Since symptomatic EBV infection doubles the risk of MS development, this virus is categorized as one of the environmental factors affecting it, and some studies have indicated that immortal EBV-induced B lymphocytes

and transformed lymphoblastoid cell lines have a fundamental role in progression of MS [22, 23]. Early studies have found that PBMCs from patients with active MS tend to transform spontaneously into lymphoblastoid cell lines and some express EBV antigens, as well. Later research studies have indicated that the difference between B-cell function in MS patients and healthy subjects is as a result of defective control of T cells that can be explained by T-cell exhaustion. In addition, molecular homology between some EBV proteins and cell death regulatory proteins hinders apoptosis in infected B cells and gives rise to loss of immune tolerance [9, 17, 24].

As reported in Figure 2, IL-2 mRNA level in MS patients was significantly higher than healthy subjects after WB treatment with both peptides. This increase illustrates the stimulation of cytokine production by the immune system which is resulting from higher activity of immune cells in MS against EBV antigens. In MS patients with EBV primary infection, T cells that have been reactivated by EBV-infected autoreactive B cells produce IL-2 and other inflammatory cytokines that attack the myelin sheath and cause common autoimmune reactions in MS [25]. In a study executed by Lünemann and colleagues [26], after treating PBMC with EBNA1 antigen, the authors reported an increase in the number of CD4⁺ T cells and the amount of IL-2. Due to similarity between myelin and EBV antigen molecules, CD4⁺ T cells attacked the myelin sheath and contributed to further development of MS.

Figure 3 illustrates a greater mRNA level of IFN- γ in EBNA1-treated MS WB, which implies that the IFN- γ -secreting immune cells of MS patients reactivated only after exposure to a latent antigen. Lünemann et al.'s [26] study has indicated an increased immune response in MS patients toward EBNA1 by evaluating the amount of secreted IFN- γ . In addition, Jilek and colleagues [27] have stated that EBV-specific CD8⁺ T cells have elevated response in early MS compared to other stages of the disease and other neurological disorders.

In many studies, EBV-specific CD8⁺ T-cell response and IFN- γ are considerably more powerful in MS patients, although in other studies, CD8⁺ T-cell response in MS is reduced or remained unchanged. This inconsistency might be the result of difference in EBV antigens, T-cell population, or MS stages researched by various studies [9, 28].

As specified in Figure 4, the cytokine secretion comparison depicts a notable difference between IL-2 and IFN- γ in the EBNA1-treated MS group, and that might be the consequence of difference in the quantity of cytokine-secreting immune cells. IL-2 and IFN- γ are substantial factors to appraise T-cell exhaustion. The primary re-

source for IL-2 production is activated CD4⁺ T cells although other immune cells such as CD8⁺ T cell have been reported to produce IL-2 [29–31]. IFN- γ is produced by NK cells, CD4⁺ T helper 1 cells, and CD8⁺ T cells [32]; however, B cells, NKT cells, and antigen-presenting cells can also secrete IFN- γ [33]. Even though IL-2 has a central role in clonal expansion, IFN- γ is thought to have influence in differentiation of CD4⁺ T cells into T helper 1 cells [34]; thus, the greater level of IL-2 might be due to higher number of CD4⁺ and CD8⁺ T cells. Moreover, IL-2 promotes effector activities in CD8⁺ T cells by inducing the expression of IFN- γ [35]. Therefore, high amount of IL-2 leads to production of IFN- γ and enhancement of CD8⁺ T-cell effector activity. Despite Pender's hypothesis, in the present study, increasing level of IL-2 and IFN- γ confirms the elevated activity of immune cells, especially T lymphocytes. These contrasting results might attribute to lengthy stimulation of cell lines whereby exhaustion took place as a consequence of prolonged exposure to EBV antigens, which is not possible in WB culture [9]. Since all of MS patients participated in this study were medicated with interferon beta-1A, it can be inferred that immune cell response was partly suppressed while it could be more intense because IFN- β , an anti-inflammatory and immune-modulatory cytokine, has antagonistic effect on proinflammatory cytokines especially IFN- γ . In addition to suppressive effect on IL-2 and IFN- γ , IFN- β diminishes T-cell activity through the cellular signaling pathway [36, 37].

On account of financial and timing limitations, this study's participants were only restricted to 20 subjects, and real-time RT-PCR was decided as the best option for cytokine evaluation, and thus the focal point of the current study was to establish an easy accessible culture (WB) for assessment of cellular cytokines. In conclusion, addi-

tional analysis of blood samples from MS patients with different medication can provide more elaborate information about the correlation between MS-specific drugs and cytokine production. Furthermore, the immune system's reaction to EBV antigenic components in MS will be more clarified by treating WB with other antigenic EBV peptides and evaluation of diverse proinflammatory cytokines.

Statement of Ethics

The research was directed ethically according to the World Medical Association Declaration of Helsinki. The study subjects have given their written informed consent, and the study protocol was approved by the Medical Ethics Committee of Tarbiat Modares University (IR.TMU.REC.1396.704).

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

Nastaran Rafiee designed and carried out the experiment, analyzed data, and wrote the manuscript. Mehrdad Ravanshad drafted and designed the experiment and supervised the research. Bahador Asadi supervised the research. Roya Kianfar performed the experiment. Ali Maleki performed the experiment and co-wrote the manuscript.

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