*Ewelina Grywalska[1, 2], Jacek Roliński[1, 2], Agnieszka Grafka[1, 2], Maciej Maj[1], Michał Mielnik[1], Marcin Pasiarski[3], Agnieszka Stelmach-Gołdyś[3], Anna Bzdek[1], Elżbieta Fitas[1], Monika Walankiewicz[1], Stanisław Góźdź[4]

An assessment of the frequency of lymphocytes with IL-4 intracellular expression and IL-4 concentration in the plasma of patients with chronic lymphocytic leukemia depending on the occurrence of Epstein-Barr virus DNA in peripheral blood mononuclear cells**

Ocena liczby limfocytów z wewnątrzkomórkową ekspresją IL-4 oraz stężenia IL-4 w osoczu u chorych na przewlekłą białeczkę limfocytową w zależności od obecności DNA wirusa Epsteina-Barr w komórkach mononuklearnych krwi obwodowej

[1]Department of Clinical Immunology and Immunotherapy, Medical University, Lublin
Head of Department: prof. Jacek Roliński, MD, PhD
[2]St. John's Cancer Center, Lublin
Head: prof. Elżbieta Starosławska, MD, PhD
[3]Department of Hematology, Holycross Cancer Center, Kielce
Head of Department: Marcin Pasiarski, MD, PhD
[4]Department of Oncology, Holycross Cancer Center, Kielce
Head of Department: Stanisław Góźdź, MD, PhD, Associate Professor

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Słowa kluczowe
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Adres/address:
*Ewelina Grywalska
Department of Clinical Immunology and Immunotherapy
Medical University of Lublin
ul. Chodźki 4a, 20-093 Lublin
tel. +48 814-486-420; 533-754-455
fax. +48 814-486-421
ewelina.grywalska@gmail.com

Summary

Introduction. Epstein-Barr virus (EBV), also referred to as human herpesvirus 4 (HHV-4), is the first identified human virus with documented involvement in carcinogenesis. Chronic lymphocytic leukemia (CLL) is a neoplastic disease characterized by clonal proliferation and accumulation of morphologically mature, albeit functionally impaired, monoclonal B lymphocytes. Despite its chronic character by definition, CLL is characterized by marked heterogeneity.

Aim. The aim of this study was to define a correlation between EBV and interleukin 4 (IL-4) in CLL patients.

Material and methods. The studies contained samples of peripheral blood obtained from 110 untreated patients with CLL. The control group was comprised of 40 healthy subjects. Peripheral blood of CLL patients and healthy controls was collected into EDTA-treated tubes. Immediately after collection, the samples were used for immunophenotyping of lymphocytes in order to assess the intracellular expression of IL-4 with the use of flow cytometry method, for isolation of mononuclear cells for the EBV-DNA copy number determination with real-time PCR method, and for plasma collection in order to the determination of IL-4 concentration with ELISA method.

Results. Patients EBV(+) had a significantly higher absolute number of CD3+/CD4+/IL-4+ and CD3+/CD8+/IL-4+ T lymphocytes, and CD19+/IL-4+ B cells than those in the control group ($p = 0.0005$, $p = 0.0006$, and $p = 0.0008$, respectively). Patients EBV(+) were characterized by significantly higher levels of IL-4 in the peripheral blood plasma than the patients EBV(-) and individuals from the control group ($p = 0.0013$ and $p = 0.0002$, respectively).

Conclusions. Our findings thus indicate that EBV-infected CLL cells produce large amounts of IL-4, which is believed to stimulate the proliferation of malignant B cells, as is the cases of lymphoproliferative diseases with known connection with EBV.

Streszczenie

Wstęp. Przewlekła białaczka limfocytowa (PBL) cechuje się heterogennym przebiegiem, którego jedną z przyczyn, w świetle najnowszych badań, mogą być czynniki zakaźne, w tym wirus Epsteina-Barr (EBV). Interleukina 4 (IL-4), będąca plejotropową cytokiną,

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której źródłem są aktywowane limfocyty T, w PBL wpływa na przeżycie białaczkowych komórek B. W literaturze światowej obecne są rozbieżne doniesienia odnośnie osoczowego stężenia i ekspresji wewnątrzkomórkowej IL-4 u chorych na PBL w porównaniu do populacji osób zdrowych.

Cel pracy. Celem pracy była ocena stężenia IL-4 w osoczu krwi obwodowej oraz jej wewnątrzkomórkowa ekspresja w limfocytach T oraz B PBL w zależności od obecności DNA EBV w komórkach mononuklearnych krwi obwodowej (PBMC).

Materiał i metody. Materiał badany stanowił próbki krwi obwodowej, pobrane od 110 nieleczonych chorych na przewlekłą białaczkę limfocytową (51 kobiet i 59 mężczyzn). Grupę kontrolną stanowiło 40 osób zdrowych, dobranych względem płci i wieku do badanych pacjentów. Niezwłocznie po pobraniu izolowano komórki mononulearne krwi obwodowej celem oceny immunofenotypu metodą cystometrii przepływowej oraz liczby kopii DNA EBV metodą real-time PCR. W uzyskanym osoczu oceniono stężenie IL-4 metodą ELISA.

Wyniki. Chorzy, u których stwierdzono obecność DNA EBV w PBMC posiadali znacząco wyższą liczbę bezwzględną limfocytów T CD3+/CD4+, T CD3+/CD8+ oraz B CD19+ z wewnątrzkomórkową ekspresją IL-4 niż osoby z grupy kontrolnej (odpowiednio: $p = 0,0005$, $p = 0,0006$ oraz $p = 0,0008$). Chorzy na PBL, u których stwierdzono obecność DNA EBV cechowali się znamiennie wyższym stężeniem IL-4 w osoczu krwi obwodowej niż chorzy z grupy EBV(-) i osoby z grupy kontrolnej (odpowiednio: $p = 0,0013$ i $p = 0,0002$).

Wnioski. Badania własne wskazują, że PBMC zakażone latentnie EBV wytwarzają znaczne ilości IL-4, która prawdopodobnie stymuluje autokrynnie i parakrynnie proliferację nowotworowych komórek B, tak jak ma to miejsce w chorobach rozrostowych o znanym związku z EBV.

INTRODUCTION

Epstein-Barr virus (EBV), also referred to as human herpesvirus 4 (HHV-4), is the first identified human virus with documented involvement in carcinogenesis (1). Epidemiological studies show that EBV-seropositive individuals constitute more than 90% of the world's population (2-4). The primary infection causes polyclonal activation and proliferation of B cells, which can manifest clinically as acute infectious mononucleosis. Subsequently, the virus enters a latent state, which may result in lifelong latent infection in the case of some hosts (5, 6). Chronic lymphocytic leukemia (CLL) is a neoplastic disease characterized by clonal proliferation and accumulation of morphologically mature, albeit functionally impaired, monoclonal B lymphocytes in lymphoid tissue, peripheral blood, bone marrow, spleen and, rarer, in other organs (7). It is estimated that CLL constitutes 25-30% of all diagnosed leukemia cases, which makes it the most prevalent malignancy of this type among adult Europeans and North Americans (8). Despite its chronic character by definition, CLL is characterized by marked heterogeneity (9, 10). Only 30% of patients survive up to 10-20 years after diagnosis (11). The remaining CLL patients develop terminal phase within 5-10 years, despite mild onset of the disease. The individuals with the aggressive form of CLL survive no more than 2-3 years after diagnosis (12). The reasons for such heterogeneous natural history of the condition remain unclear.

Potential involvement of EBV in the clinical course of CLL is still unexplained. Latent EBV infection is controlled by a cell-mediated immune response in healthy carriers. This immune response is impaired in CLL patients, and might result in poor control of reactivation and replication of the virus. Since EBV may activate B cells, stimulate their proliferation and inhibit their apoptosis, we hypothesized that it could contribute to unfavorable clinical course of CLL and may be one of the reasons for the observed disease heterogeneity. Indeed, previous studies showed that people with a history of symptomatic infectious mononucleosis, resulting from late primary EBV infection, are at increased risk of NHL. This association turned out to be the strongest for CLL, mantle cell lymphoma and B-cell promyelocytic leukemia (13).

Interleukin 4 (IL-4), which is a pleiotropic cytokine, produced mainly by the activated T lymphocytes, in CLL influences the survival of the B cells (14-17). It has been shown, that IL-4 increases the proliferation and differentiation of B lymphocytes (18). It also increases the expression of CD23 antigen, which serves as an independent negative prognostic factor in CLL (19, 20). Some authors reported an elevated mRNA level for IL-4 in T lymphocytes of CLL patients (21, 22), whereas Catellani et al. (23) in the *in vitro* experiments revealed the presence of intracellular IL-4 expression in leukemic B cells and T lymphocytes in CLL patients. Mu et al. however showed an elevated percentage of resting and activated T CD8+ lymphocytes with an intracellular IL-4 expression in patients with aggressive disease (24). Activated T CD4+ lymphocytes obtained from CLL patients also contained higher intracellular IL-4 concentration (24). According to Levesque et al. an increased production of that cytokine occurs in some CLL patients while in the others there is a reduction of its concentration in comparison to healthy donors (25). Causes of such discrepancies remain unknown.

AIM

The aim of this study was to define a correlation between EBV and IL-4 in CLL patients. The detailed objectives included determination of the EBV-DNA copy number in peripheral blood mononuclear cells (PBMC) of CLL patients and healthy individuals, and analysis of association between this parameter and IL-4 serum concentration as well as intracellular IL-4 expression in T and B lymphocytes.

MATERIAL AND METHODS

Characteristics of CLL patients and healthy volunteers

The studies contained samples of peripheral blood obtained from 110 untreated patients with CLL (51 women and 59 men) in the mean age 63.27 ± 9.73 years (median: 64, minimal age: 38, maximal age: 89 years). The control group was comprised of 40 healthy subjects (16 women and 24 men) in the mean age 64.50 ± 7.15 years (median: 64, minimal age: 53, maximal age: 79 years). Neither the CLL patients nor the controls used immunomodulating agents or hormonal preparations, showed signs of infection within the least three months prior to the study, underwent blood transfusion, or presented with autoimmune condition or allergy. Moreover, none of the controls had a history of oncological therapy or prior treatment for tuberculosis or other chronic conditions that could be associated with impaired cellular or humoral immunity.

The diagnosis of CLL was established on the basis of diagnostic criteria included in the IWCLL guidelines of the American National Cancer Institute (NCI) (26, 27). This study was approved by the Ethics Committee of the Medical University of Lublin (decision no. KE-0254/227/2010). Written informed consent was obtained from all patients with respect to the use of their blood for scientific purposes.

Examined material

Peripheral blood from the basilic vein (7 mL) of CLL patients and healthy controls was collected into EDTA-treated tubes (Sarstedt, Germany). Immediately after collection, the samples were used for immunophenotyping of lymphocytes, isolation of mononuclear cells for the EBV-DNA copy number determination, and for plasma collection in order to the determination of IL-4 concentration.

Isolation of mononuclear cells and plasma

Peripheral blood was diluted with 0.9% buffered saline (PBS) without calcium (Ca^{2+}) and magnesium (Mg^{2+}) (Biochrome AG, Germany) in 1:1 ratio. The diluted material was built up with 3 mL of Gradisol L (specific gravity 1.077 g/mL; Aqua Medica, Poland), and centrifuged in a density gradient at 700 x g for 20 min. The obtained fraction of PBMC was collected with Pasteur pipettes and washed twice in PBS without Ca^{2+} and Mg^{2+} for 5 min. Subsequently, the cells were suspended in 1 mL of PBS without Ca^{2+} and Mg^{2+}, and counted in the Neubauer chamber. Plasma was aliquoted, and stored at -80°C for enzyme-linked immunosorbent assay (ELISA).

Isolation of DNA and determination of the presence of EBV-DNA

DNA from 5 x 10^6 PBMC was isolated manually with the QIAamp DNA Blood Mini Kit (QIAGEN, Germany). The procedure for isolation followed the manufacturer's protocol. Concentration and purity of the isolated DNA were verified with the BioSpec-nano spectrophotometer (Shimadzu, Japan), on the basis of sample absorbance at 220-800 nm. The isolated DNA had good purity, with A260/280 and A260/230 absorbance ratios of approximately 2.0.

The EBV-DNA copy number in PBMC was determined with the ISEX variant of the EBV PCR kit (GeneProof, Czech Republic). Qualitative and quantitative diagnostics of EBV was performed using the Real Time Polymerase Chain Reaction (RT-PCR). An internal control in a separate tube was used. Specific conservative DNA sequence of a single-copy gene for the EBV nuclear antigen 1 (EBNA-1) was amplified in the course of the PCR process according to manufacturer's protocol. As the sensitivity of the system amounts to 10 copies/μL, all the samples with the EBV-DNA copy number below this detection threshold were considered EBV-negative [EBV(-)].

The PCR was performed with the 7300 Real Time PCR System (Applied Biosystems). The reaction was conducted on MicroAmp® Optical 96-Well Reaction Plates (Life Technologies) with MicroAmp® Optical Adhesive Film (Life Technologies).

The EBV PCR kit has successfully gone through the IVD CE marking certification for diagnostic tests. Apart from other RT-PCR systems, the kit was validated on the 7300 Real Time PCR System (Applied Biosystems) that was used in this study.

Cytometric assessment of lymphocytes with intracellular expression of IL-4

PBMC incubations were performed in 4-well plates (Nunc, Germany) for 4 hours at temperature of 37°C and the atmosphere of 5% CO_2. PBMC was added to wells (2 x 10^6 cells/mL of medium) suspended in medium, containing RPMI 1640 (PanBiotech, Germany) with 2% human albumin (Baxter, USA) and antibiotics: penicillin (100 IU/mL), streptomycin (50 ug/mL) and neomycin (100 ug/mL) (Sigma Aldrich, Germany). Apart from the medium, lymphocyte stimulators were added to the wells: PMA (Phorbol Myristate Acetate) (50 ng/mL) and ionomycin (1 ug/mL) (Sigma Aldrich, Germany). Protein transport inhibitor – brefeldine A (Sigma Aldrich, Germany) was added together with stimulators in an amount of 10 ug/mL in order to accumulate a produced cytokine in the cells. After a 4-hour incubation, collected cells were rinsed twice with PBS without Ca^{2+} and Mg^{2+} (700 x g for 5 min), and then divided in proportion of 5 x 10^5 cells/50 uL PBS for each sample tube. Prepared PBMC were marked with the following monoclonal antibodies in order to assess expression of surface antigens: anti-CD3-FITC, anti-CD4-FITC, anti-CD8-FITC and anti-CD19-FITC (BD Biosciences, USA). Cells were intubated in darkness for 20 minutes in room temperature. Subsequently they were consolidated and permeabilisated, using Cytofix/Cytoperm and Perm/Wash kit (BD Biosciences, USA), according to the manufacturer's protocol. The next step was adding 10 uL of an antibody anti-IL-4-PE (eBioscience, USA) to the corresponding tubes in order to assess the expression of

this intracellular cytokine. Then the cellular suspension was incubated for 20 minutes in the room temperature in darkness. After incubation cells were rinsed with PBS without Ca^{2+} and Mg^{2+} again (700 x g for 5 min) and immediately subjected to cytometric analysis.

Evaluation of IL-4 concentration in peripheral blood

In the blood plasma, the IL-4 concentration was determined with ELISA method. Human IL-4 Quantikine HS ELISA Kit with sensitivity of 0.22 pg/mL (R&D Systems, USA) was used. Procedure of the study was performed in accordance with the manufacturer's recommendations and an automated VICTOR3 counter (Perkin Elmer, USA) equipped with computer program WorkOut2 2.0 was used to read the records.

Statistical analysis

Normality of distribution of continuous variables was verified using the Shapiro-Wilk test. Statistical characteristics of the continuous variables are presented as median values of the lower and upper quartile, the extreme values (minimum and maximum), as well as arithmetic means and standard deviations (SD). For intergroup comparisons Student's t test for unpaired variables or U Mann-Whitney U test were used. All equations were performed using the Statistica 10 software (StatSoft, USA), taking $p < 0.05$ as a level of statistical significance.

RESULTS

In 59 patients with CLL a significant number of EBV-DNA copies were found. On this basis, the following groups were distinguished: CLL patients in whom the presence of EBV-DNA in PBMC was revealed – a group of EBV(+) patients; those who did not show the presence of EBV-DNA in PBMC – a group of EBV(-) patients and a control group.

Table 1 shows the results of evaluation of the intracellular expression of IL-4 in patients with CLL EBV(+), EBV(-) and in the control group.

Patients who revealed the presence of EBV-DNA in PBMC had a significantly higher absolute number of CD3+/CD4+ T lymphocytes with intracellular expression of IL-4 than those in the control group ($p = 0.0005$). Patients with CLL EBV(+) showed also a higher absolute number of CD3+/CD8+ T lymphocytes with intracellular IL-4 expression than those from the control group ($p = 0.0006$). These patients also were characterized by a significant increase in the absolute number of CD19+ lymphocytes with intracellular expression of the studied cytokine than healthy controls ($p = 0.0008$).

Table 2 shows the results of the evaluation of concentration of IL-4 in the peripheral blood plasma of CLL patients EBV(+) and EBV(-), and the control group.

Table 1. Statistical characteristics of intracellular expression of IL-4 in EBV(+) and EBV(-) patients and in the control group.

Variable	Group	Median	Minimum	Maximum	Mean	SD	p
The percentage of CD3+/CD4+ with intracellular expression of IL-4 [%]	EBV(+)	2.12	1.42	2.74	2.11	0.48	0.0011
	EBV(-)	0.91	0.77	1.09	0.93	0.13	
	Control	0.61	0.48	0.90	0.64	0.17	
The absolute number of CD3+/CD4+ lymphocytes with intracellular expression of IL-4 [x10^3 cells/uL]	EBV(+)	0.01395	0.00787	0.01686	0.01281	0.00371	0.0005
	EBV(-)	0.00241	0.00142	0.00476	0.00281	0.00129	
	Control	0.00007	0.00006	0.00011	0.00008	0.00002	
The percentage of CD3+/CD8+ lymphocytes with intracellular expression of IL-4 [%]	EBV(+)	1.13	0.73	1.69	1.15	0.35	0.0047 0.0494
	EBV(-)	0.95	0.62	1.17	0.93	0.19	
	Control	0.34	0.27	0.45	0.36	0.08	
The absolute number of CD3+/CD8+ lymphocytes with intracellular expression of IL-4 [x10^3 cells/uL]	EBV(+)	0.00698	0.00302	0.00936	0.00688	0.00222	0.0006
	EBV(-)	0.00261	0.00114	0.00618	0.00291	0.00174	
	Control	0.00004	0.00003	0.00006	0.00004	0.00001	
The percentage of CD3+ with intracellular expression of IL-4 [%]	EBV(+)	2.78	1.78	3.62	2.69	0.69	0.0005
	EBV(-)	1.17	0.97	1.28	1.14	0.14	
	Control	0.53	0.39	0.68	0.55	0.12	
The absolute number of CD3+ lymphocytes with intracellular expression of IL-4 [x10^3 cells/uL]	EBV(+)	0.58772	0.41367	0.86228	0.61285	0.14924	0.0006
	EBV(-)	0.27050	0.18404	0.52838	0.29958	0.13093	
	Control	0.01210	0.01071	0.01279	0.01196	0.00080	
The percentage of CD19+ with intracellular expression of IL-4 [%]	EBV(+)	2.57	1.01	4.22	2.61	1.27	0.0015
	EBV(-)	0.98	0.73	1.61	1.05	0.30	
	Control	0.54	0.20	0.90	0.51	0.28	
The absolute number of CD19+ lymphocytes with intracellular expression of IL-4 [x10^3 cells/uL]	EBV(+)	0.51052	0.33623	0.91707	0.57096	0.24639	0.0008
	EBV(-)	0.24728	0.13709	0.37564	0.25806	0.08193	
	Control	0.01021	0.00404	0.02349	0.01153	0.00723	

Table 2. The concentration of IL-4 in the peripheral blood plasma (pg/mL) in patients from groups EBV(+) and EBV(-), and the control group.

Variable	Group	Median	Minimum	Maximum	Mean	SD	p
The concentration of IL-4 in the peripheral blood plasma [pg/mL]	EBV(+)	1.06	0.29	3.72	1.21	0.93	0.0002 0.0013
	EBV(-)	0.38	0.25	0.69	0.37	0.14	
	Control	0.26	0.24	0.38	0.25	0.05	

Patients with CLL EBV(+) were characterized by significantly higher levels of IL-4 in the peripheral blood plasma than the patients EBV(-) and individuals from the control group ($p = 0.0013$ and $p = 0.0002$, respectively).

DISCUSSION

Kaminski et al. demonstrated that T cells in patients with CLL produce greater amounts of IL-4 than in the healthy subjects, what was confirmed in our own study presented in this work (28). This relationship has proved to be particularly severe in patients with CLL EBV(+), which can cause prolonged period of survival of lymphocytes in this disease. In the hereby presented studies, significantly higher levels of IL-4 in the plasma and higher absolute number of T lymphocytes (T CD3+/CD4+ and T CD3+/CD8+) and B CD19+ lymphocytes with intracellular expression of this cytokine were stated in patients EBV(+) than in the healthy subjects. IL-4 inhibits apoptosis and enhances the expression of Bcl-2 in leukemic lymphocytes, leading to the accumulation of tumor cells (29). It is worth noting that the viral protein BHRF-1 is a homologue of Bcl-2 (30, 31), so there is a possibility of influence of IL-4 on the expression of EBV proteins, which requires further investigations.

Infection of normal, resting B cells, with EBV results in the formation of lymphoblasts, which phenotypically resemble cells produced during stimulation with CD40L and IL-4 (32). It is important that as a result of infection continuously growing lymphoblastoid cell lines are forming (33), while blasts developed upon stimulation with CD40L/IL-4 have a specific survival period (34). The most recent studies have shown that EBV affects gene expression in the IL-4 dependent cellular transmitting pathways, which may be one of the most important mechanisms of oncogenesis (35). IL-4 may also have an inhibitory effect on the proliferation of leukemic B lymphocytes, as was shown already many years ago (36, 37). Inhibition of proliferation leads to the accumulation of cells in G0 phase, which predominate in CLL, and because we showed in this study significantly higher levels of IL-4 and the amount of lymphocytes producing IL-4 in patients with CLL EBV(+) compared to other groups, it seems that the relationship can be another important element in the pathogenesis of CLL.

CONCLUSIONS

Our findings indicate that EBV-infected CLL lymphocytes produce large amounts of IL-4, which is believed to stimulate the proliferation of malignant B cells, as is the cases of lymphoproliferative diseases with known connection with EBV.

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