Rituximab in the Treatment of EBV-positive Low Grade B-Cell Lymphoma

PANAGIOTIS T. DIAMANTOPOULOS[1], KATERINA POLONYFI[1], MARIA SOFOTASIOU[1], VASILIKI PAPADOPOULOU[1], FANI KALALA[1], THEODOROS ILIAKIS[1], KOSTANTINOS ZERVAKIS[1], GERASSIMOS TSILIMIDOS[1], PANAGIOTIS KOUZIS[1], MARIE-CHRISTINE KYRTSONIS[2], THEODOROS VASSILAKOPOULOS[2], MARIA ANGELOPOULOU[2], MARINA SIAKANTARIS[1], GEORGE VAYOPOULOS[1], PANAGOULA KOLLIA[3], GERASSIMOS PANGALIS[2] and NORA-ATHINA VINIOU[1]

[1]*Hematology Unit, First Department of Internal Medicine, and*
[2]*Department of Hematology, Laikon General Hospital, National and Kapodistrian University of Athens, Athens, Greece;*
[3]*Department of Genetics and Biotechnology, Faculty of Biology,*
School of Physical Sciences, National and Kapodistrian University of Athens, Athens, Greece

Abstract. *Background: Following infection of B lymphocytes by Epstein Barr virus (EBV), the viral genome remains in the nucleus, and a latency phase is established, during which only a small proportion of the viral genes are expressed. Among them, LMP1 is essential for transformation. Rituximab is a potent agent used in the treatment of low grade B-cell lymphomas and is also widely used for the treatment of post-transplant lymphoproliferative disorders caused by EBV. The effect of rituximab treatment on the latent EBV infection in non-transplant patients with lymphoproliferative disorders has never been studied to our knowledge. Patients and Methods: We studied, the effect of rituximab-based immunochemotherapy on the EBV status of 44 patients with leukemic low grade B-cell lymphoma. Results: After three cycles of rituximab-based treatment, only 1/17 patients was still positive for EBV. Discussion: Our results suggest that rituximab used in the treatment of EBV-positive low-grade lymphoma is efficient in eradicating the virus from the peripheral blood, a fact with potential implications in the course and prognosis of the disease.*

Epstein Barr virus (EBV) is a ubiquitous pathogenic DNA virus that is transmitted through close contact of asymptomatic carriers and susceptible individuals. The vast majority of infections are sub-clinical, and the detection of antibodies against viral antigens is a common finding in all population groups worldwide.

Like other members of the human herpesvirus family, EBV demonstrates a latent phase of infection. Infection of B-lymphocytes usually results in latency that is characterized by incorporation of the viral genes into the host genome and selective expression of a restricted subset of viral products (1).

In humans, B-lymphocytes, T-lymphocytes, epithelial cells and myocytes are the host cells for EBV. Susceptible cells bear the receptor for EBV on their surface. On the surface of B cells this receptor is CD21, which is the receptor of complement component 3b (C3b), or complement receptor 2 (CR2) (2). It is currently believed that glycoprotein 350 (gp350) of the virus binds exclusively to CD21, and this is the inaugural event for the infection of B cells (3). CD21 antigen is also present on the surface of T-cells, although structural differences may exist (4). *In vitro* studies have shown that the virus is also capable of infecting monocytes (5). After their infection, monocytes present reduced phagocytic function, and these cells may serve as a site of early virus proliferation and may be responsible for blunting immune response against the virus.

The hallmark of B-cell infection by EBV is the establishment of latency. Latency is characterized by three distinct processes: viral persistence, restricted viral gene expression and retained potential for re-activation of the lytic cycle (6). Only 10 out of about one hundred viral genes (six nuclear proteins, two latent membrane proteins and two EBV encoded RNAs) are expressed during latency and are considered to be implicated in establishing and maintaining malignant transformation (7). Latent membrane protein 1 (LMP1), among them, is highly stable and encodes an integral membrane protein. It has been shown that LMP1 is

Correspondence to: Nora-Athina Viniou, MD, Ph.D., Hematology Unit, First Department of Internal Medicine Laikon General Hospital, National and Kapodistrian University of Athens, Athens 11527, Greece. Tel: +30 2107456843, Fax: +30 2107788830, e-mail: noravi@med.uoa.gr

Key Words: Epstein Barr virus, low-grade B-cell lymphoma, rituximab.

essential for EBV-induced transformation of B-cells and protects EBV-infected B cells from apoptosis, in part *via* induction of the cellular oncogene BCL2 (8). LMP1 is a potent activator of Nuclear Factor–kappaB (NF-kB), through two domains within its carboxy-terminal part, C-terminus-activationg region 1 (CTAR1) and 2 (CTAR2) (9), by engaging signaling proteins for the tumor necrosis factor-associated factors (TRAFs) (10). Due to these multiple potent biological effects, LMP1 has become a potential target in EBV-associated tumor prevention and treatment.

Rituximab (a chimeric monoclonal antibody against CD20) is the mainstay of treatment for EBV related post-transplant lymphoproliferative disorder (PTLD). As far as we are aware of, the effect of treatment with rituximab on EBV viral status has never been studied in EBV-positive non-transplant patients with lymphoproliferative disorders. Rituximab is a common choice for treatment of patients with low grade B-cell lymphomas and has been recently approved for B-cell chronic lymphocytic leukemia (B-CLL) (11-13), either as monotherapy or in combination with chemotherapy. The effect of rituximab on the EBV status of such patients is not absolutely predictable, especially because rituximab is implicated in the re-activation of viral infections in patients with lymphoproliferative disorders. The results of rituximab administration in patients with PTLD indicate that this medication serves as an antiviral factor (14) but these results cannot be extrapolated to other patient groups.

In the present study, we detected EBV positivity, quantified the viral load, and detected *LMP1* mRNA and protein in patients with low-grade B-cell lymphoma before treatment with immunochemotherapy that included rituximab. After three cycles of rituximab administration we again measured the viral load, and *LMP1* mRNA and protein in order to compare pre- and post-treatment results.

Patients and Methods

Patients. Forty-four patients with non-EBV-related leukemic low grade B-cell lymphoma were included in the study. Informed consent was obtained from all patients. Table I shows the epidemiological and clinical characteristics of the patients, as well as the patients' distribution by lymphoproliferative disease according to the WHO classification of tumours of hematopoietic and lymphoid tissues (15). The diagnoses were established in each case using clinical, morphological, histopathological, and immunophenotypic criteria. All patients had immunophenotypically-confirmed disease by peripheral blood at the time of sample collection. All patients were due to be treated with immunochemotherapy that included rituximab, according to common clinical practice. The programmed and eventually administered treatment schemes are shown in Table I. Blood samples were collected before the first rituximab administration and after three treatment cycles. The rituximab dose used was 375 mg/m^2 in monthly cycles. We obtained peripheral whole blood samples from all patients. All samples were collected in ethylenediaminetetraacetic acid (EDTA).

DNA extraction. Two hundred microliters of whole blood from each sample were used for DNA extraction. Extraction was performed by using the QIAamp® DNA Blood mini kit (Qiagen, Valencia, CA, USA) and DNA was eluted in 35 μL double-distilled water according to the manufacturer's instructions, within six hours from collection. Extracts were then stored at –20˚C and analyzed as described below.

RNA extraction and cDNA synthesis. The Trizol protocol (Invitrogen, Carlsbad, CA, USA) was used to extract and purify total RNA from peripheral whole blood samples. Total RNA was eluted in 30 μl of ribonuclease-free water. RNA was quantified spectrophotometrically and its quality was tested by electrophoresis. For each patient sample, 1 μg of RNA, 200 units M-MLV reverse transcriptase (RT) (Invitrogen) and 100 pmol/μl hexamer random primers were used to synthesize cDNA after an incubation of the reaction mix at 37˚C for 50 minutes.

EBV-viral capsid antigen (VCA) IgG detection. The serological status of EBV was determined by a chemiluminescent immunoassay [CLIA; the Liaison (CLIA-L; DiaSorin)] which detects IgG antibodies against the EVB VCA.

EBV load quantification. The quantification of the viral load was based on the detection of the bxlf-1 thymidine kinase gene of the virus using DNA from whole blood samples. For this purpose, the EBV R-gene™ Quantification Complete kit (Argene, Verniolle, France) was used according to the manufacturer's instructions. Quantification was performed on a LightCycler® 2.0 instrument (Roche, Mannheim, Germany). Reactions were carried out in duplicates and negative and sensitivity controls were also used. For quantification analysis, an internal standard curve was applied for each run. The detection limit of the reaction was <4 copies/PCR. The results were expressed in viral copies/ml of sample.

Primer design. Primers and probes for Real Time Polymerase Chain Reaction (RT-PCR) for the genes of interest were chosen for *LMP1* (16) and Abelson murine leukemia viral oncogene (*ABL*) (17) in order to bind at least one exon junction so as to avoid amplification of contaminating genomic DNA.

Conventional RT-PCR. LMP1-mRNA expression was detected by conventional RT-PCR. The stably expressed housekeeping gene *ABL* was used as an endogenous control. The PCR for *LMP1* and *ABL* genes was carried out in a gradient cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) using 1 unit of Platinum-Taq DNA polymerase (Invitrogen), 1 μl of cDNA, 0.4 μl of each primer (forward and reverse, 0.2 μM each), 0.7 μl of $MgCl_2$ (1.4 mM) for *LMP1* and 0.75 μL of $MgCl_2$ (1.5 mM) (Invitrogen) for *ABL* and finally 0.5 μl of dNTPs (0.2 mM).

The thermal profile of the reaction was as follows: initial denaturation for 5 minutes at 95˚C, followed by 45 cycles of denaturation at 95˚C for 45 seconds, annealing at 58˚C for 45 seconds (for *LMP1*) and at 61˚C for 45 seconds for *ABL*, and extension at 72˚C for 45 seconds, and lastly one cycle of final extension at 72˚C for 10 minutes. Each reaction contained template-negative and reverse transcriptase negative samples as controls, as well as one positive and one negative for the *LMP1* cell line control. A human cell line (MDA-V) was used as a positive control for LMP1 detection. MDA-V is a novel cell line (a kind gift from Dr. R. Ford, The University of Texas M. D. Anderson Cancer Center,

Table I. *Patient characteristics and results*

Characteristic	All patients	EBV status[1]		*p*-Value
		Positive	Negative	
No. of patients	44 (100%)	17 (38.6)	27 (61.4)	
Median age, years (range)	74 (51-87)	71.5 (51-87)	75.5 (55-82)	0.372
Male: female ratio	1.09	1.71	0.81	0.250
Lymphoproliferative disease, N (%)				0.126
B-Chronic lymphocytic leukemia	29 (65.9)	15 (88.2)	14 (51.9)	
Splenic marginal zone lymphoma	12 (27.3)	1 (5.9)	11 (40.7)	
Hairy cell leukemia	2 (4.5)	1 (5.9)	1 (3.45)	
Follicular lymphoma	1 (2.3)	0 (0)	1 (3.7)	
B Symptoms at presentation, n (%)	6 (13.6)	3 (17.6)	3 (11.1)	0.538
Peripheral blood lymphocytes, $\times 10^9$/l (range)	21.14 (1.39-81.03)	28.1 (2.95-81.03)	15.1 (1.39-40.80)	0.127
LDH/UNL at presentation, mean (range)	1.62 (0.7-3.3)	1.55 (0.8-3.0)	1.71 (0.7-3.3)	0.584
Seropositivity[2], n (%)	42 (95.4)	16 (94.1)	26 (96.3)	0.656
Previous treatment, n (%)	17 (38.6)	7 (41.2)	10 (37.0)	0.730
Median time from previous treatment, months (range)	16.79 (2-80)	12.14 (6-29)	19.50 (2-80)	0.633
Previous treatment containing rituximab, n (%)	5 (11.4)	2 (11.8)	3 (11.1)	0.912
Median viral load before rituximab-based treatment, copies/ml (range)	NA	2375.3 (79-15600)	NA	
Measurable LMP1 mRNA before rituximab-based treatment, n (%)	11 (25.0)	11 (64.7)	0 (0)	
Detection of LMP1 in the serum (ELISA) before treatment, n (%)	11 (25.0)	11 (64.7)	0 (0)	
Immunochemotherapeutic regimen, n (%)				0.213
R	20 (45.5)	10 (58.8)	10 (37.0)	
RCh	9 (20.5)	2 (11.8)	7 (25.9)	
FCR	6 (13.6)	4 (23.5)	2 (7.4)	
R-C(H)OP	7 (15.9)	1 (5.9)	6 (22.2)	
RCy	1 (2.3)	0 (0)	1 (3.7)	
R-FCM	1 (2.3)	0 (0)	1 (3.7)	
Patients with measurable viral load after rituximab-based treatment, n (%)	NA	1 (5.9)	NA	

*For group differences by Pearson Chi-Squared test for categorical data, and by independent samples Mann-Whitney *U*-test for numerical data; [1]By status of bxlf1 thymidine kinase gene of EBV by real time Polymerase chain reaction; [2]Seropositivity: detection of the EBV-VCA (viral capsid antigen) IgG antibody. UNL: Upper normal limit; R: rituximab RC: Rituximab, chlorambucil; FCR: fludarabin, cyclophosphamide, rituximab; R-C(H)OP: rituximab, vincristine, cyclophosphamide, (doxorubicin), prednisolone; R-FCM: rituximab, fludarabine, cyclophosphamide, mitoxantrone; NA: not applicable.

Houston, TX, USA) that has been established and characterized at our Institution. MDA-V was established from lymph nodes of a previously untreated patient with EBV-positive stage I classical nodular sclerosing Hodgkin's lymphoma(16). Karpas 299, an anaplastic large-cell lymphoma cell line was used as a negative control. The amplified PCR products were electrophoresed and visualized twice on a 3% agarose gel. The *LMP1* PCR product of 102 bp was verified with direct sequencing.

Detection of LMP1 in serum by enzyme-linked immunosorbent assay (ELISA). LMP1 expression in the sera of all *LMP1*-positive patients (positive by PCR for LMP1 mRNA) was assessed with the use of ELISA (LMP1 detection kit, MYBiosource, San Diego, CA, USA).

Statistical analysis. We used the Independent-Samples Mann-Whitney *U*-test (IBM SPSS statistics, version 19.0) for the statistical analysis of the results.

Results

The vast majority of the patients (29/44, 65.9%) had B-CLL. Among 44 patients, 17 (38.6%) were found to be EBV-positive by qRT-PCR for the *bxlf*-1 gene. The mean viral load of EBV-positive patients was 2,375.3 (range=79-15,600) copies/ml. Eleven (64.71%) EBV-positive patients had measurable *LMP1*-mRNA levels, and this result was confirmed by ELISA for the detection of LMP1 protein in the serum of these patients. Details are presented in Table I.

The distribution by lymphoproliferative disease did not differ significantly (p=0.126) between EBV-positive and EBV-negative patients, although in the EBV-negative group, splenic marginal zone lymphoma seems to be over-represented (40.7% *vs*. 5.9% in the EBV-positive group).

Twenty seven out of 44 (61.4%) patients were treatment-naïve. Only 2/17 (11.8%) EBV-positive and 3/27 (11.1%) EBV-negative patients had been previously treated with rituximab and this treatment had been administered at least six months before sample collection. Moreover, 1/17 (5.9%) EBV positive and 2/27 (7.4%) EBV-negative patients had been previously treated with regimens containing fludarabine. None of the patients had been previously treated with alemtuzumab.

Following sample collection, all patients were treated with rituximab-based immunochemotherapy; the used treatment regimens are displayed in Table I. There was no statistically significant difference in the treatment regimens between EBV-positive and EBV-negative patients (p=0.213). After three cycles of rituximab-based treatment, only 1/17 patient (5.88%) was still positive for EBV and expressing LMP1. This patient with B-CLL was treated with a regimen containing fludarabine, cyclophosphamide and rituximab, but did not achieve hematologic response. This failure was accompanied by an increase in the EBV viral load.

Discussion

Although treatment with rituximab, through B-cell depletion, is a cause of viral re-activation [hepatitis B and C virus (18, 19), cytomegalovirus, varicella-zoster virus and others (20)] in immunocompromised and hematological patients, this agent has been widely used in the treatment of EBV-related PTLD.

PTLD is a well-characterized complication of organ (21, 22) and bone marrow transplantation (23, 24). In most cases, PTLD is associated with EBV infection due to either re-activation of the virus in an already infected transplant recipient, or a primary infection acquired from the donor. Measurement of EBV-DNA levels in the peripheral blood appears to be a useful predictor for the development of PTLD prior to the onset of clinical disease (25), thus enabling for early therapeutic intervention. The management of EBV-associated PTLD consists of reduction or withdrawal of immunosuppressive agents, and rituximab administration alone or in combination with low-dose chemotherapeutic regimens (14, 26-28). Four cycles of rituximab usually precede the administration of chemotherapy (29-31). Preemptive administration of rituximab in patients developing significant EBV viremia has been proven safe and highly effective in preventing PTLD in transplant recipients (32, 33).

The rationale for rituximab administration in EBV-related PTLD is based, among others, on the successive expression of CD20 and CD21 antigens on the surface of B-cells. CD20 is a phosphoprotein expressed on the surface of all B-cells beginning at the pro-B phase (CD45R+, CD117+) and progressively increasing in concentration until maturity (34). CD21, the receptor of EBV, is present on all mature B-cells. This means that all CD21-positive cells are also CD20-positive. Rituximab destroys cells bearing CD20 antigen, and consequently CD21-positive cells, that is, cells that host the virus or are susceptible to viral infection.

The ability of the virus to infect both monocytes and T-lymphocytes may be a cause of incomplete eradication of the virus in patients with latent EBV infection and lymphoproliferative disease. Monocytes, in particular, may serve as a reservoir for EBV during B-cell depletion caused by rituximab (35-37).

Our results are in concordance with the already known action of rituximab in PTLDs. After three cycles of rituximab-based immunochemotherapy only one out of 17 EBV-positive patients was found to be positive by quantitative RT-PCR for the bxlf1 gene of the virus. This patient was a 65-year-old man; he had been diagnosed 16 months earlier with B-CLL (Rai stage IV at diagnosis), had bulky disease, no hypoglobulinemia, and had not been treated in the past with rituximab or fludarabine. His pre-treatment viral load was 3,250 copies/ml, but post-treatment analysis revealed a substantial increase (5,300 copies/ml). In this patient, the failure to eliminate EBV from the peripheral blood was accompanied with treatment failure. Bulky disease is an adverse prognostic factor for patients with lymphoproliferative diseases, and it remains as such even in the rituximab era, due to the limited efficacy of rituximab in the treatment of such patients (38, 39). Moreover, latent EBV infection in patients with B-CLL has been linked to poorer prognosis, in part because of its correlation with Richter's transformation (40). The remaining 16 patients experienced at least a partial remission of their disease, along with having an undetectable EBV viral load.

Our results indicate that the use of rituximab is not a risk factor for EBV re-activation in non-transplant EBV-positive patients. Moreover, our study suggests that the use of rituximab as part of the treatment of EBV-positive patients with low grade B-cell lymphoma may result in EBV eradication from the peripheral blood, a fact with various possible implications. For example, EBV-positive patients are at risk of fatal complications due to EBV re-activation when treated with heavily immunosuppressive regimens, such as those containing fludarabine (41-43), or alemtuzumab (44-47), a fact that is probably underestimated. The addition of rituximab to fludarabine and cyclophosphamide in patients with CLL may also serve as antiviral protection against EBV. On the other hand, pre-treatment of EBV-positive patients with rituximab could be a measure for EBV eradication, before alemtuzumab administration. In addition, the viral load of the virus could be a good factor predictive of disease unresponsiveness or recurrence in patients that have a measurable pretreatment viral load.

The extension of this preliminary study, with the inclusion of more patients, and the comparative study of rituximab versus other chemotherapeutic regimens in terms of EBV infection will give more accurate results about the role of rituximab in EBV-positive lymphoproliferative disorders.

Conflicts of Interest

None declared.

Source of Funding

Non declared.

Acknowledgements

We would like to express our gratitude to Professor Michael Koutsilieris Professor of Experimental Physiology, National and Kapodistrian University of Athens, Greece, for his valuable critique of this research work.

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Received October 2, 2013
Revised November 9, 2013
Accepted November 12, 2013