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Detection of specific lytic and latent transcripts can help to predict the status of Epstein–Barr virus infection in transplant recipients with high virus load★

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Epstein–Barr virus (EBV), a member of the family *Herpesviridae*, is widely spread in the human population and has the ability to establish lifelong latent infection. In immunocompetent individuals the virus reactivation is usually harmless and unnoticeable. In immunocompromised patients productive infection or type III latency may lead to EBV-associated post-transplant lymphoproliferative disorder (PTLD). The aim of our research was to investigate the utility of PCR-based methods in the diagnosis and monitoring of EBV infections in bone marrow transplant recipients. Thirty-eight peripheral blood leukocyte samples obtained from 16 patients were analysed, in which EBV DNA was confirmed by PCR. We used semi-quantitative PCR to estimate the viral load and reverse-transcription PCR (RT-PCR) to differentiate between latent and productive EBV infection. In 14 patients we confirmed productive viral infection. We observed a correlation between higher number of EBV genome copies and the presence of transcripts specific for type III latency as well as clinical symptoms.

Keywords: Epstein-Barr virus, latency, EBV-lymphoproliferative disorder, productive infection, bone marrow transplantation

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous herpesvirus widely distributed in the human population. Primary infection is asymptomatic during early childhood. However, in adolescents or in young adults, nearly half of the cases of primary infections manifest as mononucleosis. EBV infects mainly B lymphocytes and certain epithelial (lymphoepithelial) cells of oropharynx. Like other herpesviruses, EBV has the ability to establish lifelong latent infection in circulating B lymphocytes, so that the virus reactivation can occur, but in immunocompetent individuals it is usually harmless and symptomless (Rickinson & Kieff, 2007). Nevertheless, EBV infection may be life–threatening in immunocompromised patients such as bone marrow or solid organ transplant recipients (Wagner *et al*., 2002; Tsurumi *et al*., 2005; Rickinson & Kieff, 2007). In these patients EBV reactivation may lead to persistent lytic infection with high viral load in peripheral blood lymphocytes. EBV-driven B cells can proliferate and progress to immunoblastic lymphoma which is associated with type III EBV latency. In such cases the mortality rate is very high

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Abbreviations: BALF-2, early gene of Epstein–Barr virus; PBL, peripheral blood leukocytes; EBNA, Epstein–Barr virus nuclear antigen; BZLF-1, immediate-early gene of Epstein–Barr virus; EBV, Epstein–Barr virus; GvHD, graft *versus* host disease; HHV7, Human Herpesvirus type 7; HSCT, haematopoietic stem cell transplantation; LMP, latent membrane protein; M-MLV, Molonay murine leukemia virus; PTLD, post-transplant lymphoproliferative disorder.

and reaches 50 to 80% (Wagner *et al*., 2002). Early identification of patients at risk for developing EBV-associated post-transplant lymphoproliferative disorder (PTLD) could reduce morbidity and mortality, thereby improving overall patient management (Rezonable & Paya, 2003).

Numerous studies have confirmed the correlation between high EBV viral load in peripheral blood and the risk of developing PTLD (Savoie *et al*., 1994; Bai *et al*., 2000; Jebbink *et al*., 2003; Wagner *et al*., 2004). However, quantitative methods give no information about the stage of EBV infection. Lytic or productive infection is characterized by expression of >60 early and capsid structural genes, whereas in the immortalized or continuously proliferating state, typical for type III latency, transcripts for the EBNAs and LMPs genes are present. Therefore, owing to a possibility of developing a severe viral disease, rapid, reliable and highly sensitive diagnostic methods for monitoring EBV infection are needed.

The aim of our research was to investigate the usefulness of polymerase chain reaction (PCR) — based methods: RT-PCR and semi-quantitative PCR, in the diagnosis and monitoring of bone marrow transplant recipients. The main purpose of the applied methods was the detection of EBV transcripts characteristic for lytic as well as type III latency, estimation of viral genome copy number and correlation of the results with patients' clinical status after transplantation.

MATERIALS AND METHODS

Samples. We studied 38 samples of peripheral blood leukocytes (PBL) from 16 EBV-positive patients after allogenic haematopoietic stem cell transplantation (HSCT) in different stages of engraftment. Prospective monitoring of our patients has shown that in some recipients EBV loads have fluctuated while in others persisted for more than 3 months. All analysed patients have undergone pre-emptive antiviral therapy with acyclovir or gancyclovir. In our research we also used Namalwa cells (Burkitt lymphoma cell line) as a positive control. Namalwa cells contain two copies of EBV DNA incorporated into the genome (Vernard *et al*., 2000). As a negative clinical control we used leukocytes obtained from eight EBV-negative patients (confirmed in serological as well as in PCR tests) who had undergone allo-HSCT.

RNA and DNA extraction. Eukocytes obtained from recipients were isolated by sedimentation of 3–5 ml EDTA-treated blood samples with 6% dextran solution according to the method described by The *et al.* (1995). Aliquots of 1×10^6 PBL or Namalwa cells were used for RNA extraction by Trizol Reagent (1 ml of Trizol Reagent (Invitrogen), per 100 µl of cell suspension) and DNA extraction using Genomic DNA Prep Plus (A&A Biotechnology).

Viral DNA assay by nested PCR. To confirm the presence of EBV-1 or EBV-2, two-step PCR for EBNA2 was applied according to Venard *et al*. (2000) (Table 1). In EBV-positive samples the amount of EBV DNA was confirmed by a semi-quantitative PCR. Ten-fold dilutions of a DNA standard isolated from 1×10^6 Namalwa cells were amplified by PCR and separated by gel electrophoresis. DNA samples from patients were also diluted 10-fold, amplified, and the bands separated on a gel were compared to the standard run in parallel. The calculated EBV load for all samples was expressed as the number of EBV genome copies per 10^6 PBL (Fig. 1A).

Viral mRNA assay by RT-PCR. Reverse transcription of RNA samples was performed using oligo-dT primers (Sigma) and Mo-MLV reverse transcriptase (Sigma). The efficiency of RT reaction was verified by PCR for β-actin (Table 1). cDNA samples were used to detect specific EBV transcripts by PCR. All PCR products were separated by electrophoresis and the intensity of the bands was compared and marked as "+", "++", or "+++" (Fig. 1B).

Primers were chosen to distinguish between EBV productive and latent infection. For that pur-

Table 1. Targets for primers used in PCR and RT-PCR

	Stage of infection:	Gene/transcript	Product	According to:
House-keeping gene	–	Beta-actin	307 bp	Guzik *et al.*, 1999
EBV	Confirmation of infection	EBNA2/EBV1 or 2	497 bp or 150 bp	Vernard *et al.*, 2000
	Productive infection	BZLF1	608 bp	Mundle *et al.*, 2001
		BALF2	238 bp 118 bp	Prang *et al.*, 1997
	Type III latency	EBNA2	596 bp 497 bp	Venard *et al.*, 2000
		LMP1	351 bp 198 bp	Qu *et al.*, 2000

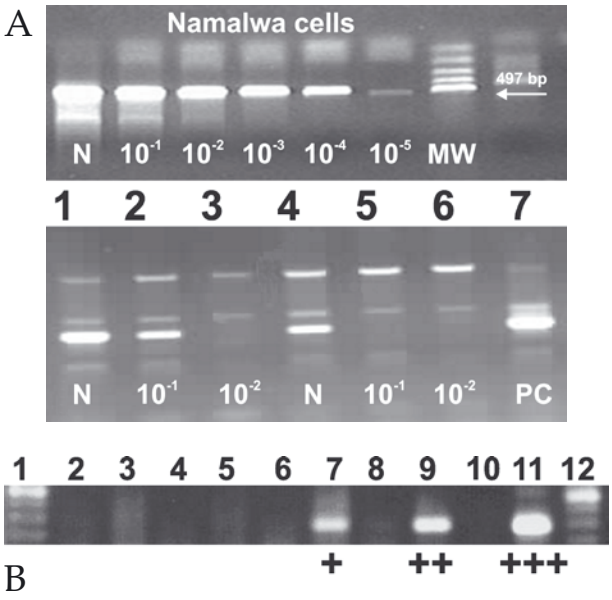

Figure 1. Examples of gel electrophoresis pattern of PCR products.
A. PCR for EBNA2/EBV1. Serial dilution of DNA obtained from 10^6 Namalwa cells (upper panel) and DNA obtained from two selected patients' samples (bands no. 1–3 and 4–6). PC, positive control, DNA isolated from Namalwa cell line (band no. 7). **B**. RT-PCR for LMP1 showing different intensity of gel bands; bands no. 1 and 12, molecular marker, no. 2–10 selected samples, no. 11, positive control from Namalwa cells.

pose, transcripts of productive infection: BZLF1 (immediate-early) and BALF2 (early) and type III latency transcripts for viral oncogenes — EBNA2 and LMP1 — were detected (Table 1). We performed two-step PCRs (nested PCRs) for detection of BALF2, EBNA2 and LMP1 transcripts to increase the sensitivity of the method. PCR for BZLF1 transcript was one-step.

RESULTS

The results obtained for blood samples of EBV-positive patients are presented in Table 2. The presence of EBV-1 was confirmed in all 38 samples of PBL. Basing on the serological tests before transplantation in the donor–recipients pairs, primary EBV-infection could be recognized in two patients (no. 12 and 13), while in the others the presence of EBV-1 was a result of reactivation or possibly reinfection. Samples were collected at different stages of engraftment (from one to 29 months after HSCT).

Isolated RNA was analysed by RT-PCR to differentiate between productive and latent EBV infection and mostly to detect transcripts specific for type III latency, connected with a higher risk for developing PTLD. In all studied samples the transcript for the house-keeping gene β-actin was detected, which confirmed the efficiency of RNA extraction and RT reaction. Moreover, all specific transcripts were present in the Namalwa cell line used as a positive control. In no material obtained from uninfected patients, serving as a negative clinical control (results not shown in Table 2), did we detect any EBV transcripts.

Productive infection was detected in 14 of 16 analysed patients (patients no. 3–16). Almost in all of the samples (83%) a transcript for the *BALF2* gene (essential in the lytic phase of the viral replication) was detected, while a transcript for the immediate-early gene *BZLF1* was detected only in 19% of materials obtained from four patients (patients no. 6, 9, 12 and 13). It is possible that the difference in the number of positive samples for productive infection of EBV is a consequence of the different sensitivity of one-step and two-step PCR. It is also possible that the detection of further stages of EBV lytic infection (i.e. for late genes) may be much more useful in the diagnostics of bone marrow transplant recipients.

Transcripts for viral oncogenes EBNA2 and LMP1, specific for type III latency of EBV, were detected in 5 of 16 patients (patients no. 4, 9, 12, 13 and 15) although expression of the *BZLF1* and/or *BALF2* genes, characteristic for lytic infection, was also confirmed. In patients no. 1 and 2 only LMP1 transcript was present, which confirmed the latent stage of EBV infection, but not type III latency.

The aim of the semi-quantitative PCR analysis was to estimate the number of EBV viral genome copies in the DNA samples studied. A number of EBV genome copies (between 2000 and 20000 per 1×10^6 leukocytes) was demonstrated for 8 out of 16 patients, and in six of them (patients no. 3, 4, 12, 13, 14 and 15) the higher viral load correlated with symptoms such as graft *versus* host disease (GvHD), increased levels of transaminases, thrombocytopenia, and fever. For patient no. 15, between 7 to 9 months after transplantation, we observed an increase of the EBV copy number from 200 to 20 000 per 1×10^6 PBL without clinical symptoms (data not included in Table 2). Furthermore, samples of patient no. 14 collected after 1.5 and 2 months following HSCT were only positive for the BALF2 transcript specific for productive infection and the estimated EBV genome copy number was low – 200 per 1×10^6 PBL. Because in these sample the presence of HHV7 was also detected, the high level of transaminases may be a result of a mixed infection. In the sample collected from the same patient 3 months after transplantation, we additionally confirmed the LMP1 transcript that accompanied an increase of the EBV genome number to 2000 per 1×10^6 PBL and the appearance of thrombocytopenia.

Table 2. Results of RT–PCR and semi–quantitative PCR compared with clinical symptoms

Patient [sex/age]	Serostatus^ D/R	Time after HSCT [months]	EBV copy no/10^6PBL	Detected EBV transcripts				Symptoms and additional herpesvirus infection
				Productive infection		Type III latency		
				BZLF1*	BALF2**	EBNA2**	LMP1**	
1 [F/20]	–/+	8	200	–	–	–	+	chronic GvHD, liver and kidney failure
2 [F/21]	–/+	6	200	–	–	–	+	no symptoms
3 [F/36]	+/+	2.5	2 000	–	+	–	–	thrombocytopenia, CMV infection
		17	200	–	–	–	+ +	no symptoms
4 [M/35]	+/+	3	200	–	–	–	+	no symptoms
		19	2 000	–	+ +	+ +	+ +	GvHD, increased level of transaminases
5 [F/23]	+/+	29	200	–	+	–	–	increased level of transaminases thrombocytopenia, HHV–7 infection
6 [M/39]	+/+	16	200	–	+	–	–	no symptoms
		18.5	200	–	+	–	–	
		19	200	+	+	–	–	
7 [M/22]	+/+	1	200	–	+	–	–	no symptoms
		2.5	200	–	+	–	–	
8 [F/27]	–/+	2.5	200	–	+ +	–	–	no symptoms
		3.5	200	–	+	–	–	
		4.5	200	–	+	–	–	
		5	200	–	+	–	–	
9 [M/23]	+/+	2	200	–	+	–	–	no symptoms
		3	200	+	–	+++	+	
		4	<200	–	+	–	+	
10 [F/43]	–/+	6	200	–	+	–	–	relapse of malignancy
11 [M/55]	+/+	1.5	2 000	–	+ +		–	no symptoms
12 [M/35]	–/–	4.5	20 000	+	–	–	–	chronic GvHD, fever, increased level of transaminases, thrombocytopenia
		6.5	2 000	+ +	+ +	+ +	+ + +	
		7	2 000	+ +	+ +	+ +	+ +	
		17.5	200	–	+	–	–	

13	–/–	3	2 000	–	+ +	–	+ +	
[M/24]		4.5	20 000	–	+	–	–	GvHD, increased level of transaminases,
		6	2 000	–	+	–	+ +	thrombocytopenia, HHV6 infection
		11	2 000	–	+	–	+	relapse of malignancy
		14	2 000	+	–	+ + +	+	CMV infection
		14.5	2 000	+	+ + +	+ + +	+	
		15.5	200	–	–	–	–	
14	+/+	1.5	200	–	+ +	–	–	increased level of transaminases,
[F/34]		2	200	–	+	–	–	HHV7 infection
		3	2 000	–	+ +	–	+	thrombocytopenia
15	+/+	9	20 000	–	+ +	+ +	+ +	increased level of transaminases
[M/22]								
16	–/+	9.5	2 000	–	+	–	–	chronic GvHD
[F/36]		10	200	–	+ + +	–	–	

^, presence of EBV antibody in donor (D) and recipient (R); *, one-step PCR **, two-step PCR (nested PCR); +, ++; +++. intensity of band fluorescence

A low number of EBV genome copies, 200 per 1×10^6 PBL, was usually found in samples of "healthy" recipients. The increasing viral load observed in patient no. 4, as well as the decreasing EBV load in patient no. 3 correlated well with their clinical status.

In our study patients were examined for EBV transcripts between 1 and 29 months after HSCT. For most of the patients, samples were collected within a year, on average 4.7±2.8 months after HSCT, and also later, except for patients no. 5 and 6, whose samples were examined only late after transplantation. The highest risk of developing PTLD has been reported for the first year after transplantation, when the recipients' effective cytoxic immune response against EBV-infected B lymphocytes was recovering (Bhatia *et al*., 1996). In our patients we did not observe such complication, neither have we found typical type III latency, without a productive gene expression. However, in samples with reasonably high EBV copy number (> 200 per 10^6 PBL) the EBNA2 and/or LMP1 transcripts, characteristic for type III latency, were detected significantly more often, at 67% *vs*. 30% in low-load samples (Fisher exact test, P=0.03).

DISCUSSION

Developing reliable and highly sensitive methods for Epstein–Barr virus detection is crucial for the growing number of immunosuppressed patients. In immunocompetent seropositive hosts, EBV is controlled by cell-mediated immunity and the presence of viral genome is usually undetectable in PBL (Hopwood *et al*., 2002). Immunosuppressive therapy may increase the number of peripheral EBV-driven B cells. In our allo-HSCT patients we observed transient EBV appearance in blood cells. From our studied group three patients (no. 9, 12 and 13) were persistent EBV carriers for more than 3 months. In one carrier, low copy number (200 copies/10^6 PBL) was detected, whereas in two others a high viral load was observed as a result of primary infection. Increasing virus load was associated with clinical symptoms. Because for some patients we had confirmed other herpesviruses in the same samples (Zawilinska *et al*., 2006), it cannot be concluded definitively that these symptoms were caused by EBV only.

In most reports EBV DNA levels in patients with PTDL are significantly higher than in healthy recipients (Hopwood *et al*., 2002). In our patients the highest level of virus loads was 20 000 copies per 10^6 PBL although in none of them PTDL was observed. So we additionally analysed the expression of specific EBV transcripts which reflected EBV productive infection or latency. Progressive features, ranging

from reactive hyperplasia to monomorphic B cell lymphoma (Nalesnik, 1998; Barrett, 2000), difficulties in PTLD diagnosis and usually a fatal outcome of the disorder (Wagner *et al*., 2002) are reasons for the application of new diagnostic methods for detection of EBV infection and PTLD.

In our investigation we were able to detect productive infection in the majority of studied patients (even asymptomatic ones) in contrast to Bergallo and co-workers who confirmed such infection in only one of 30 asymptomatic renal recipients (Bergallo *et al*., 2007). The results of our study indicate also that there is a correlation between the higher number of EBV genome copies and the presence of transcripts specific for type III latent infection. It may be proposed that the appearance of EBV-infected B lymphocytes with type III EBV latency can induce excessive viral replication, or that an increased number of lytic EBV-infected B cells results in the expression of type III latent genes in other infected lymphocytes. The quantitative methods more often applied in the diagnosis of EBV infection, such as real-time PCR (Savoie *et al*., 1994; Bai *et al*., 2000; Jebbink *et al*., 2003; Wagner *et al*., 2004), can be useful in predicting the risk of developing PTLD, even though the risk is directly related to EBV latent infection in contrast to other herpesviruses, in which case the productive phase of the life cycle causes virus-associated diseases in transplant recipients (Rayes *et al*., 2005).

To conclude, these preliminary results indicate that PCR-based assays seem to be useful in the monitoring of EBV infection in bone marrow transplant recipients. Semi-quantitative PCR is regarded as a valuable tool for estimating EBV genome copy number and also allows evaluation of the risk for patients, since a high amount of EBV DNA is one of the major risk factors for developing PTLD. However, our results and another study show that an increased viral load in transplant recipients is not always predictive of PTDL (Tysarowski *et al*., 2007). Using the RT-PCR method in our study additionally allowed us to differentiate between productive and latent infection. Therefore, application of several methods, rather than only one, for monitoring transplant recipients seems to be more reliable and gives much more information about EBV infection. Early identification of patients at risk for developing PTLD could reduce PTLD-related morbidity and mortality by appropriate patient management.

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