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Lymphotropic herpesvirus DNA detection in patients with active CMV infection – a possible role in the course of CMV infection after hematopoietic stem cell transplantation

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The natural history of cytomegalovirus (CMV) infection and disease in transplant recipients prompts researchers to look for other factors contributing to this infection. The ubiquity of lymphotropic herpesviruses (EBV, HHV-6, and HHV-7) and the possibility of their activation during immunosuppression may suggest their participation in progression of CMV infection in patients after hematopoietic stem cell transplantation (HSCT).

Material/Methods: The presence of CMV, EBV, HHV-6 and HHV-7 was confirmed through detection of viral DNA isolated from leukocytes. Allo-HSCT recipients (n=55) were examined repeatedly within the average period of 14±7.3 months post-transplant.

Results: CMV DNA was detected in 24% of samples, while EBV, HHV-6 and HHV-7 were detected in 20%, 15% and 14% of samples, respectively. Based on the presence of CMV infection at particular timepoints (months) after transplantation, the recipients were divided into 3 groups: Group I (N=15) with persistent infection, Group II (N=20) with transient infection, and Group III (N=20) without CMV infection. In Group I, the mean CMV load was significantly higher than in Group II, and the clinical condition of Group I patients was poorer. All these patients manifested clinical symptoms, and all had episodes of GvHD. All Group I patients developed multiple infections; EBV in 80%, HHV-6 in 47% and HHV-7 in 87% of patients. In the remaining groups, with the exception of HHV-6 in group II, the frequency of infected patients was lower. In addition, CMV presence was often preceded by another herpesvirus.

Conclusions: The results suggest that other herpesviruses, mainly HHV-7, could predispose CMV to cause chronic infection.

key words: bone marrow transplantation • CMV DNA load • herpesviruses mixed infections

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Background

In the last few years hematopoietic stem cell transplantation [HSCT] has become the preferred treatment of congenital and acquired diseases of the hematopoietic system, as well as other diseases such as lymphoproliferative disorders, solid tumors, autoimmune diseases or immune deficiencies [1–3]. Despite the expansion of this method of treatment, and continuing evolution of technology, there is still a serious risk of infectious complications after transplantation, varying depending on the type of HSCT, the underlying disease severity, conditioning regimens, prophylaxis applied and post-transplant complications. The risk and type of infection is various at different times post-transplantation [4,5].

Human cytomegalovirus (CMV) continues to be one of the most important pathogens in immunocompromised patients such as bone marrow or solid organ recipients and those with AIDS [6,7]. Among these patients CMV may cause a variety of clinical manifestations, from mild illness to severe, life-threatening diseases, which follow a primary or recurrent acute infection. Asymptomatic chronic CMV infection may be associated with graft dysfunction, as well as atherosclerotic vascular lesions [8–10]. The outcome of CMV infection may be related to the humoral and cellular immune status of the patient. In transplant patients, the possibility of active infection depends on the serostatus of the donor/recipient, the type of transplantation and regimen of immunosuppressive therapy [7]. In cases of recurrent or persistent CMV infection, the probability of reinfections with different CMV strains or of resistance as a consequence of extended antiviral therapy must be considered. Recently, considerable attention has been given to research of cofactors that might increase the severity of CMV infection and affect CMV replication. One of these cofactors may be a multiple infection, especially with other herpesviruses [4,11,12].

We studied the possible association between CMV replication, the outcome of CMV infection and other beta herpesviruses, such as human herpesvirus-6 (HHV-6), human herpesvirus-7 (HHV-7), and Epstein-Barr virus (EBV) in patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The above-mentioned viruses were selected in view of the frequency of such infections in a healthy adult population, their capacity for latency and reactivation, and the similar route of transmission after HSCT transplantation. Among these studied viruses only CMV is routinely monitored after transplantation. The presence of DNA of all the 4 herpesviruses (HHV-6, HHV-7, EBV, and CMV) in HSCT patients was detected with polymerase chain reaction (PCR) techniques. The kinetics of the appearance of particular herpesviruses after transplantation, not only in the early, but also in the late period, more than 6 months post-transplant was analyzed.

Material and Methods

Study population

Fifty-five recipients of allo-HSCT (23 females and 32 males, average age 31.8±10 years), in whom follow-up could be carried out for at least 6 months after transplantation, were enrolled into the study. The transplantations were performed at the Department of Hematology, Jagiellonian University Medical College, between 2002 and 2008. The reasons for transplantation were lymphoid (14 acute, 1 chronic) or myeloid (23 acute, 9 chronic) leukemias. Eight patients received transplants for other diseases. Twenty-eight patients received peripheral blood stem cells, and 27 patients received bone marrow cells, although within this group 2 of them additionally underwent peripheral stem cell transplantations. All patients received transplants from related donors. Acute graft-*versus*-host disease (GvHD) was prophylaxed with cyclosporine and methotrexate. Acute GvHD and chronic GvHD were diagnosed based on clinical evaluation and graded according to the Seattle criteria [13].

The patients were given standard prophylaxis for bacterial infections (ciprofloxacin, 500 mg twice daily p.o.), fungal infections (fluconazole, 400 mg daily p.o.), and viral infections (acyclovir, 800 mg twice daily p.o.). No patients received prophylactic intravenous immunoglobulins, ganciclovir, foscarnet, or cidofovir.

For over the first 3 months following transplantation the patients were weekly, prospectively monitored for CMV infection, and subsequently when CMV infection was suspected. CMV infection and disease were defined according to the published recommendations [14]. CMV-related symptoms were classified as viral syndrome if the patient showed unexplained fever for more than 2 days accompanied by active CMV infection, and 1 of the following symptoms: leucopenia ($<3\times10^9$/L), thrombocytopenia ($<100\times10^9$/L), or arthralgia. The viral syndrome plus pneumonia, hepatitis, gastroenteritis, or central nervous system involvement was defined as CMV disease. Active CMV infection was determined based on the detection of pp65 antigen in leukocytes (>2/200 000 cells) and/or the presence of CMV DNA in 2 consecutive blood samples. These results were obtained as part of routine surveillance used for clinical management. In these cases, antiviral pre-emptive therapy was based on intravenous infusion of either ganciclovir at 10 mg/kg/day or foscarnet at 60–90 mg/kg/day for 2 to 3 weeks. Patients with evidence of CMV disease were treated with ganciclovir 5 mg/kg of body weight twice a day for 2 weeks and then given maintenance therapy 5 mg/kg of body weight daily or 5 times per week for 4 weeks, depending on the level of immunosuppression and the absolute neutrophil count. Some patients (n = 3) were treated with cidofovir at dose 5 mg/kg administered once every 2 weeks.

Detection of EBV, HHV-6 and HHV-7 DNA was carried out only retrospectively and did not influence the diagnosis or patient management. In collected samples with confirmed CMV or HHV-6 presence, the viral load was also quantified using real-time PCR (RT-PCR). EBV-positive samples were evaluated by semi-quantitative PCR.

The study was approved by the Local Ethics Committee and performed following the principles of the declaration of Helsinki.

CMV and EBV serology

CMV and EBV serostatus was ascertained in all the donors and recipients before transplantation, using a commercially

available enzyme-linked immunoassay (Dade Behring), according to the manufacturer's instructions.

Specimen processing

Peripheral blood leukocytes (PBLs) obtained from the recipients were isolated by sedimentation of 3–5 ml EDTA-treated blood samples with 6% dextran in phosphate-buffered saline (PBS) according to the method described by The et al [15]. Suspension of 2×10^5 leukocytes per slide were used to prepare cytospins. The slides were fixed in 5% buffered formalin for antigenemia pp65 assay. The remaining leukocytes (1×10^6 cells) were used for DNA extraction, using the Genomic DNA Prep Plus kit (A & A Biotechnology, Poland).

CMV detection

The CMV pp65 antigenemia test was carried out using fluorescent-labeled monoclonal antibodies NCL–HCMV pp65 (Novocastra) according to Gerna et al. [16]. The results were reported as the number of positive cells/200 000 leukocytes.

CMV DNA detection was performed using primers specific for viral glycoprotein B (gB) described by Mitchel et al [17]. The "outer" set was 5'-GTCGACGGTGGAGATACTGCTGAGG-3' and 5'- GAGGACAACGAAATCCTGTTGGGCA-3', which amplifies a 150 bp sequence. The "inner" primer pair was 5'-ACCACCGCACTGAGGAATGTCAG-3' and 5'-TCAATCATGCGTTTGAGGAGGTA-3', which amplifies a 100 bp sequence. PCR reactions were run in the thermocycler (Biometra) in a total volume of 50 µl consisting of 2 µl of sample DNA or control solutions in water, 10 mM Tris-HCl pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 1 U of Taq polymerase DyNAzyme™ (Finnezymes); 200 µM dNTP and 100 ng of each primer. The amplification program included a 5-minute denaturation step at 94°C followed by 35 cycles of 2 minutes at 95°C, 2 minutes at 58°C (for external primers) or 50°C (for internal primers) and 1 minute at 72°C. A final elongation step at 72°C took 7 minutes. The presence of Taq DNA polymerase inhibitors was checked by adding 2 ng of DNA (obtained from a cell culture infected with the AD-169 strain of HCMV) to the negative samples and retesting. The PCR products were visualized by electrophoresis in 2% agarose gel with ethidium bromide.

CMV DNA was quantified using the Q-CMV Real-Time Complete kit (Nanogen Advanced Diagnostics). The amplification reaction was specific for the MIEA gene of CMV and for the region of the human beta-globin gene (as an internal control of inhibition). The probes specific for CMV and beta-globin were fluorophore-labeled with FAM and VIC, respectively. The reaction was performed in the Applied Biosystems 7500 Fast Real-Time PCR System according the manufacturer's instructions. The results were calculated as the CMV genomes equivalent/million cells.

EBV detection

To confirm the presence of EBV-1 or EBV-2, 2-step PCR for the EBNA2 gene was applied according to Venard et al. [18]. Five microliters of DNA isolated from clinical samples or Burkitt lymphoma cell line (Namalwa) as a positive control were used for amplification of 596 bp segment common to EBV-1 and EBV-2. A total of 30 cycles (each cycle consisted of 94°C for 1.5 minutes, 60°C for 1 minutes and 72°C for 2 minutes) were carried out. A second run used 2 separate sets of primers specific for EBV-1 and EBV-2 amplified fragments of 497 bp or 150 bp, respectively. PCR products were analyzed in 2% agarose gel in TBE buffer containing ethidium bromide. In EBV-positive samples, the amount of EBV DNA was confirmed by a semi-quantitative PCR [19].

Amplification of HHV-6 and HHV-7 genomes

HHV-6 DNA was detected by nested PCR amplifying a consensus sequence of the major capsid protein encoding region for both HHV-6 variants A and B, as described previously [20]. A total volume of 50 µl reaction mixture containing 5 µl of the sample, 200 µM dNTP, 1 U *Taq* polymerase and 0.125 µM of each external primer in PCR buffer was heated at 95°C for 5 minutes (initial denaturation) and followed by 30 cycles of 1 minute each at 95°C, 55°C and 72°C, with 10 minutes of final extension at 72°C. Five microliters of the product from the first PCR were amplified in a second reaction under the same conditions, except 0.25 µM of internal primers was used. PCR products were visualized by electrophoresis, and those that amplified this region were used to assess the type of HHV-6. To distinguish between A and B variants of HHV-6, a set of variant-specific nPCR assays was applied according to Yalcin et al. [21]. Samples positive for HHV-6 were quantified with the commercial HHV-6 real-time PCR kit (Nanogen Advanced Diagnostics). The amplification reaction was specific for the OFR 13R region of HHV-6 and for the region of the human beta-globin gene (as an internal control of inhibition). The specific probes for the virus and beta-globin were fluorophore-labeled with FAM and VIC, respectively. The results were calculated as the HHV-6 genomes equivalent/million cells.

HHV-7 DNA was detected by nPCR with primers described by Chan et al. [20]. The final product was a fragment of 124 bp. Both rounds of amplification were performed in 50 µl containing of 1×PCR buffer, 200 µM dNTP, 1 U *Taq* polymerase and 0.125 µM primers. The template volume was 5 µl of DNA in both rounds. An initial denaturation at 94°C for 5 minutes was followed by 40 cycles of 1 minute each at 94°C, 54°C (first round) or 48°C (second round), and 72°C for extension. The final elongation step was extended to 10 minutes at 72°C. The PCR product of the second round was determined by gel electrophoresis.

With each run of nPCR, a negative no-template control and positive controls of HHV-6 and HHV-7 (DNA obtained from clinical samples of patients with previously confirmed infection) were included.

Statistical analysis

Descriptive statistics were used to calculate the incidence of viral infections. The results were expressed as mean or median ±SD. Continuous variables were analyzed by the Mann-Whitney U test, with values of $p<0.050$ considered significant. Dichotomous variables were analyzed using the chi-square test or Fisher's exact test. Comparison of viral kinetics in particular groups was carried out by the non-parametric Wilcoxon's test. The statistical analysis was done using the STATISTICA PL 8.0 software package.

RESULTS

Frequency of CMV infection

Out of 55 patients that had undergone allo-HSCT, 45 were pre-transplant seropositive; 39 of them obtained cells from CMV-positive donors, and 6 CMV-seronegative patients received mismatch transplantations. Active CMV infections were confirmed in 35 recipients, in whom we were able to detect CMV DNA in nested PCR and quantified using a commercial real-time PCR test.

In this part of the study, 1386 samples obtained from 55 HSCT recipients were tested. The presence of CMV was confirmed in 331 (23.9%) leukocyte samples. Based on CMV detection in PBLs and its kinetics after transplantation, the recipients of allo-HSCT were divided into 3 groups: Group I – 15 recipients with persistent (maintained for >3 months) and recurrent (>5 episodes) CMV infections; Group II – 20 patients with sporadic (<3 episodes) and transient CMV infections during the entire follow-up period; and Group III – 20 patients without CMV infections. The characteristic features of patients in each group according to CMV results are presented in Table 1. In Group I, CMV was significantly more frequent in PBLs than in Group II, in 44.4% *vs.* 20.6% samples, respectively. The difference of CMV loads in Group I and Group II was also significant.

EBV, HHV-6 and HHV-7 DNA detection

Detection of EBV, HHV-6 and HHV-7 was performed in selected samples, usually collected once to twice per month during the follow-up. Based on the observation of fluctuation of positive and negative results in consequent samples obtained from the same patients, we concluded that detection of viral DNA was a result of viral active replication, not latency. In analyzed positive samples for EBV or HHV-6, only EBV type 1 or HHV-6 variant B were confirmed.

Serological examinations before transplantation had shown that the majority of transplant recipients were EBV-seropositive, only 5 persons were seronegative (2 in Group II and 3 in Group III), and all of them had obtained cell-grafts from EBV-seronegative donors. After transplantation, EBV DNA was detected in 12 (80%) patients in Group I, in 13 (65%) patients in Group II and in 10 (50%) recipients in Group III (Figure 1). The presence of HHV-6 DNA was confirmed in 7 (47%) patients in Group I, 13 (65%) in Group II, and in 7 (35%) in Group III. The detection pattern of HHV-7 in patients belonging to the above-mentioned groups was as follows: 13 (87%), 7 (35%) and 5 (25%), respectively (Figure 1). The incidence of HHV-7 infection in Group I was statistically more frequent than in Group II and Group III (Fisher's exact test, p=0.0027 and p=0.0004, respectively). No such relationship was observed for the remaining viruses investigated.

The results of DNA detection of the studied viruses (EBV, HHV-6 and HHV-7) with respect to the number of tested samples in 3 groups of patients are presented in Table 2. EBV was detected more frequently than other viruses (Chi^2 test, p=0.01). The average number of EBV-positive PBL samples was 2.7±3.3 per patient. The analysis of the frequency of EBV DNA in leukocyte samples in particular groups of patients demonstrated a significantly higher percentage in Groups II and III as compared to Group I (Chi^2 test, p=0.0035 and p=0.0116, respectively), which may be explained by appearance of EBV primary infection in patients in these groups. The mean EBV load was determined as 2.73±0.67 $\log_{10}$ genomes/million cells, range: 2.3–5.5, and it was comparable in the studied groups.

The presence of HHV-6 and HHV-7 in the total number of tested samples was apparently similar – 14.8% and 13.6%, respectively (Table 2). The mean number of DNA-positive samples per person for HHV-6 and HHV-7 was also similar – 1.5±2.6 and 1.4±2.4, respectively. The comparison of HHV-7 positive samples in Groups I to III revealed that HHV-7 DNA was more frequent in Group I than in Groups II and III, and these differences were statistically significant (Chi^2 test, p<0.0001 and p=0.0013, respectively). Quantification of HHV-6 DNA in real-time PCR showed a low load of HHV-6 in the tested samples, with the mean value of 0.82±0.68 $\log_{10}$ genomes/million cells, range: 0.3–2.28. The mean loads in particular groups were comparable and the differences were not significant (Mann-Whitney U test).

Correlation between CMV and other herpesviruses

Out of 558 samples examined concurrently for 4 viruses, 86 (15.41%) were positive for at least 2 viruses. Co-infections by double viruses predominated (88.4%). Co-infections were detected with the least frequency in Group III (5.3% of samples), while in Group I and Group II, they were observed with a similar frequency, in 21.8% and 17.3% samples, respectively. These frequency rates were significant in comparison to Group III (Chi^2 test, p<0.00001 and p=0.0005, respectively).

The appearance of particular viruses following transplantation is shown in Figure 2, in which the relative frequency of viruses adjusted to the same number of samples examined for each virus was used. This approach was necessary because in the first months after transplantation, the patients were monitored more frequently as compared to the later period. Detection of CMV predominated in the early months after allo-HSCT; however, in late period CMV also appeared in both Group I and Group II. The statistical analysis of differences in CMV DNA detection in consecutive months after transplantation was significant in Group I and Group II (Wilcoxon's test, p=0.0023). A similar relationship in Group I and Group II was observed for HHV-7 (Wilcoxon's test, p=0.0052), and also for Group I and Group III (Wilcoxon's test, p=0.0131). The kinetics of HHV-6 appearance was significantly different between Group II and Group III (Wilcoxon's test, p=0.0159).

Table 3 shows the sequence of appearance of studied viruses in patients in the following months after transplantation. CMV or HHV-6 appeared earlier than others. The mean time to initial detection of HHV-7 and EBV was significantly longer and was comparable for each virus, also in particular groups of patients. Because in the majority of patients herpesviruses appeared sequentially after transplantation, patients infected with CMV were analyzed in order to establish a hypothetical relation between CMV and other viruses (Table 4). The virus that was most

Table 1. Characteristics of patients after allo-HSCT grouped according to CMV DNA results in PBLs.

	Group I (n=15)	Group II (n=20)	Group III (n=20)	p-Value*
Gender (M/F)	10/5	9/11	13/7	
Age at transplant (years)				
Median ±SD	36±11.5	32±8.6	32±11.0	ns
Range	18–55	19–44	17–58	
Diagnosis				
Acute lymphoblastic leukemia	4	5	5	
Chronic lymphocytic leukemia	–	–	1	
Acute myeloid leukemia	7	10	6	
Chronic myeloid leukemia	2	4	3	
Lymphoma	–	1	–	
Multiple myeloma	1	–	–	
Severe aplastic anemia	–	–	3	
Other diseases	1	–	2	
Source of stem cells				
Peripheral blood	7	13	8	
Bone marrow	7	7	11	
Bone marrow + peripheral cells	1	–	1	
CMV serostatus (donor/recipient)				
Positive/positive	13	12	14	
Positive/negative	1	4	1	
Negative/negative	–	2	2	
Negative/positive	1	2	3	
Time of follow-up (months)				
Median ±SD	14.0±9.6	9.5±5.6	12.5±5.4	0.02 (I *vs.* II)
Range	8–35	6–23	6–23	
Results of CMV study:				
No samples tested	545	432	409	
No CMV DNA positive (%)	242 (44.4)	89 (20.6)	0	<0.00001
Mean No pos. samples/patient ±SD	16.1±5.3	4.5±1.9		<0.00001
Mean CMV load** ±SD	2.83±0.78	2.39±0.96		0.012
Range**	1.28–4.54	0.48–4.53		
Median CMV load**	2.90	2.35		

Group I – patients with maintained CMV infection, Group II – patients with sporadic CMV infection, Group III – patients without CMV infection. PBL – peripheral blood leukocytes; SD – standard deviation; ns – non-significant; * – Chi-squared or Mann Whitney U test as appropriate; ** – $\log_{10}$ of CMV genomes equivalent/million PBLs.

commonly detected as the first one was HHV-6, while in the second run it was CMV, and in the third run it was EBV. Differences between Group I and Group II were not statistically significant.

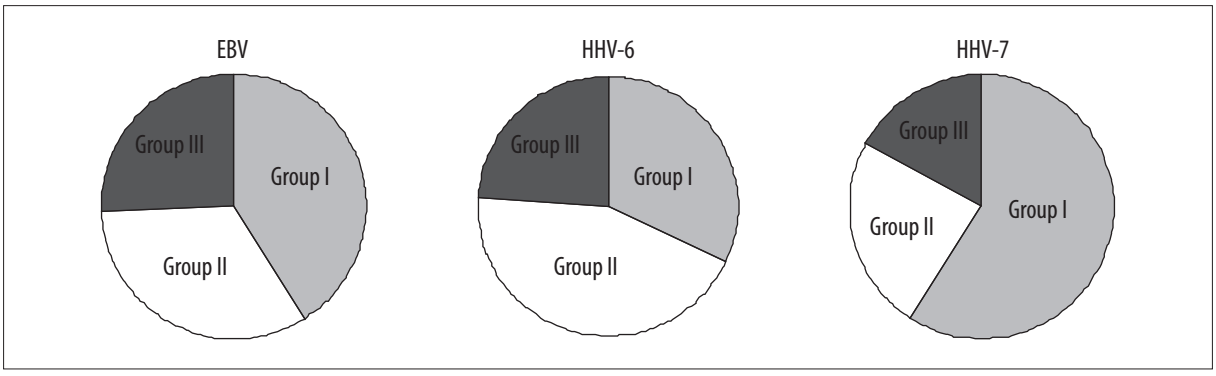

Figure 1. Distribution of EBV, HHV-6 and HHV-7 in patients belonging to various groups.

Table 2. Incidence of EBV, HHV-6 and HHV-7 DNA detection in samples obtained from allo-HSCT patients in our study groups.

Virus	No. tested*	% positive	Group I No. tested (% pos.)		Group II No. tested (% pos.)		Group III No. tested (% pos.)	
EBV	742	20.08	270	(14.07)	244	(24.18)	228	(22.81)
HHV-6	568	14.79	210	(14.29)	186	(21.51)	172	(8.14)
HHV-7	558	13.62	209	(22.49)	179	(6.70)	170	(10.00)

* The differences of number samples in Group I and Group II as well as in Group II and III are not significant (Chi-square test).

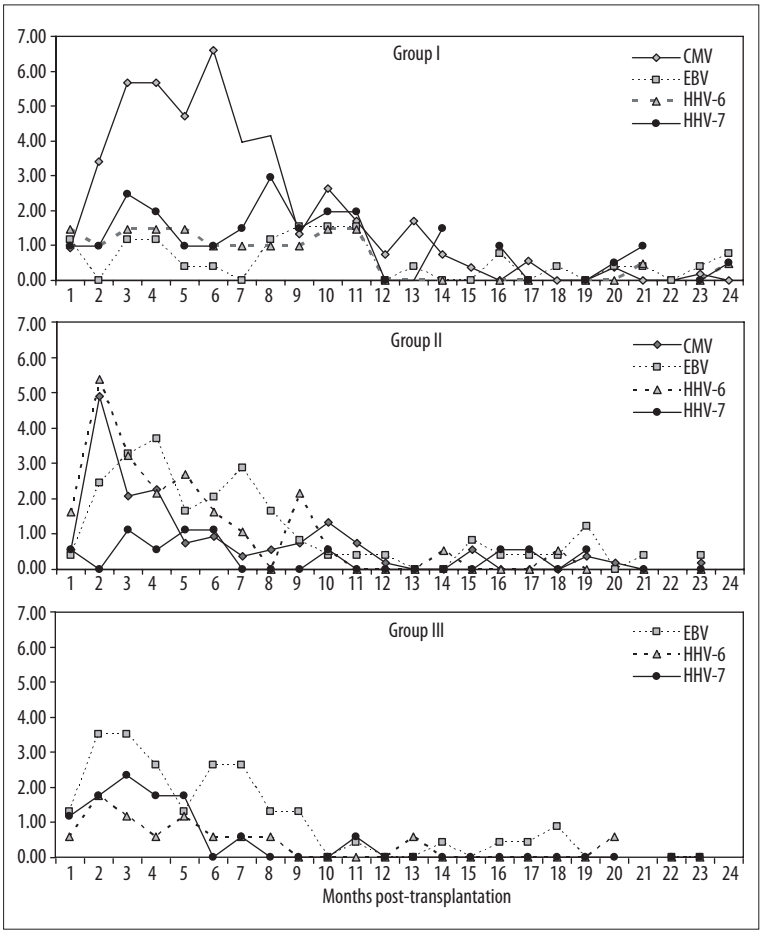

Figure 2. Relative frequency of CMV, EBV, HHV-6 and HHV-7 DNA in peripheral blood leukocytes post-transplant.

Table 3. Time from transplantation to detection of first positive sample (in days) in patients after allo-HSCT.

Virus	No. of positive patients	Days from HSCT median ±SD	Range	95% CI for mean value	p-Value*
HHV-6	26	46.0±134	11–597	50–159	ns
CMV	35	53.0±39	19–217	46–73	
HHV-7	24	75.5±135	10–468	75–189	0.05
EBV	35	94.0±133	19–621	85–176	0.025

* Mann Whitney U test; comparison for CMV; CI – confidence interval; ns – non-significant.

Table 4. Sequence of herpesviruses appearance following allo-HSCT in 32 CMV-positive patients with multiple infections. The table shows the number and percentage (in brackets) of patients with positive samples.

Sequence	CMV n=32	EBV n=25	HHV-6 n=20	HHV-7 n=20	p-Value*
• First	14 (43.8)	8 (32.0)	11 **(55.0)**	5 (25.0)	ns
• Second	15 **(46.9)**	5 (20.0)	6 (30.0)	7 (35.0)	0.03 for EBV
• Third	3 (9.4)	11 **(44.0)**	3 (15.0)	7 (35.0)	0.003 for EBV, 0.03 for HHV-7
• Fourth	0	1 (4.0)	0	1 (5.0)	ns

* Fisher' exact test, comparison to CMV frequency; ns – non-significant.

Correlation between viral infection and clinical presentation

The clinical presentation was different in patients belong to particular groups (Table 5). It should be emphasized that any viral DNA was detected in 5 individuals from Group III; in 9 patients of this group, only single infections were recognized, while double infections were seen in 5 patients and a triple infection in 1 patient. In Group II, only 3 patients had singular CMV infections, while 3-virus infections predominated. In Group I, all the patients had mixed viral infections, caused by 3 (13 patients) or 4 viruses. The patients in Group I developed infections with the frequencies of 3.13±0.35 viruses as compared to 2.65±0.99 and 1.10±0.85 viruses in Group II and Group III, respectively. These differences were statistically significant for Group I *vs.* III, as well as for Group II *vs.* Group III (the Mann-Whitney U test, p=0.000002 and p=0.00007, respectively). The clinical condition of Group I patients was poorer than in the remaining groups. The indirect exponent of this phenomenon was a longer time of follow-up and a higher frequency of sample testing in Group I (Tables 1, 2). Liver dysfunction was predominant in all patients. Aminotransferase levels (ALT and ASP) showed mean values that exceeded normal levels 6±4 – fold in Group I, 4±4 – fold in Group II and 3±1 – fold in Group III. These differences in aminotransferases levels were statistically significant for Group I as compared to Group II and Group III (the Mann-Whitney U test, p=0.05). Severe GvHD occurred in all the patients belonging to Group I, while in Group II and Group III, this complication was observed in 40% and 10% of recipients, respectively (Fisher's exact test, p=0.0002 and p<0.0001).

Discussion

In long-term follow-up of allo-HSCT recipients (a half of studied patients were tested for over a year after transplantation), CMV, HHV-6, and HHV-7 frequently occurred in our study. CMV DNA was present in nearly 24% of the tested samples, and EBV-1 DNA was present in 20%. HHV-6 B and HHV-7 were detected with a somewhat lower frequency, in 15% and 14% of the samples, respectively. In the present study, DNA isolated from leukocytes was used; however, other researchers prefer plasma for herpesvirus DNA detection to eliminate the possibility of amplification of latent forms of viral genome [11,22,23]. In the present study, productive infections were confirmed using specific CMV transcripts for productive and latent infection (data not shown). This was based on results of a previous study [24]. Razonable et al. [25], in their quantitative assay simultaneously performed in DNA isolated from whole blood, plasma, PBLs and blood mononuclear cells, demonstrated the adequacy of all blood compartments for CMV monitoring in transplant recipients. In our opinion, although the use of PBLs was more time-consuming and technically more difficult than using whole blood, it allowed us to detect and compare the presence of specific viral DNA in the same leukocyte numbers during reconstitution of the graft. In our research, using PCR method for CMV, EBV, HHV-6 and HHV-7 detection, fluctuations of positive and negative results in consecutive samples in the same patient were observed. The load of EBV and HHV-6 DNA was relatively low, considering the most dangerous complications following transplantation as a post-transplant lymphoproliferative disease (PTDL) and HHV-6 meningoencephalitis. As the exponent of EBV and HHV-6 relative low loads was that none of the studied patients had

Table 5. Clinical presentation of viral infections and GvHD in patients belonging to various groups.

	Group I	Group II	Group III
Asymptomatic infection	0	5	11
Symptomatic infection:	15	15	4#
• Fever	2	–	1
• Thrombocytopenia	8	5	2
• Leucopenia	8	3	2
• Anemia	1	1	–
• Gastoenteritis	3 (1*)	2**	–
• Hepatitis	1*	–	–
• Increased aminotransferases	13	10***	3
• Pneumonia	1*	1	–
• Retinitis	1	–	–
GvHD	15	8**	2
• Acute	7	3	2
• Chronic	13	6	2
Chronic GvHD manifestation:			
liver	8	2	1
skin	7	1	1
gastrointestinal tract	3	1	
joint	3	2	
conjunctiva	1		

* CMV confirmed in autopsy; ** in one patient with a double EBV + HHV6 infection; *** in 3 cases associated only with EBV or HHV-6 infection; # EBV primary infection (1 patient), EBV+HHV-7 (2 patients) and EBV+HHV-6 (1 patient).

been affected by the lymphoproliferative syndrome associated with EBV and nobody suffered from HHV-6 encephalitis. Moreover, the low load of HHV-6 found in our study shows that none of the patients had chromosomal integration of HHV-6. The chromosomal integration of HHV-6 found in 0.2–2.9% of asymptomatic individuals is characterized by persistent abnormally high viral level [26,27].

The present study allows us to recognize multiple infections in the majority of allo-HSCT patients (69%): 10 subjects were infected by double viruses, 22 by triple viruses, and 6 recipients by 4 viruses. These mixed infections were detected retrospectively. Both the American Society of Transplantation Infectious Diseases Guidelines and other authors of the VICTOR trial who carried out observations on a large cohort of patients after solid organ transplantation have suggested that routine monitoring for viral co-infections in patients with CMV disease is not indicated [11]. Our observations on HSCT recipients, however, confirmed frequent herpesvirus mixed infections.

In our study, all patients were prospectively monitored for CMV infection only, according to the guidelines for HSCT patients and, based on positive results, they received antiviral treatment. Even though all patients were administered antiviral agents for a similar period, differences in CMV-clearance in some patients were observed. Because of this we consider that other viruses have an influence on the course of CMV infection. Based on the presence of CMV infection and its kinetics, the patients were divided into 3 groups and were studied for other herpesviruses that could potentially promote replication and persistence of CMV. In Group I, severe complications and persistence of CMV DNAemia or high rates of its recurrence were observed. Out of 20 CMV-infected recipients in Group II, only 10 had CMV infections, but their response to antiviral treatment was better. In this group, a prompt decrease of CMV DNA was noted, and recurrences were seen rarely in follow-up. In Group III, CMV DNA was not detected.

In our retrospective study, materials previously collected were used to search for other lymphotropic herpesviruses that have the potential to be pathogenic in transplant recipients. Based on PCR, EBV was detected in 64% patients, in the same proportion as CMV, followed by HHV-6 (47%) and HHV-7 (44%). These results were similar to previous reports focusing on hematopoietic stem cell or solid organ transplant patients [22,28–31]. Chan et al. [28] investigated bone marrow transplant recipients, and reported CMV in 61%, HHV-6 in 28% and HHV-7 in 53% of the individuals, while Kidd et al. [29] researched renal transplant patients and observed parallel viruses in 58%, 23% and 46% of the patients. A high percentage of infected patients were also recorded by Wang et al. [30], and this is in agreement with the observations presented here, especially with respect to CMV and EBV incidence. Other authors also reported frequent HHV-6 and HHV-7 infections in adult transplant recipients, describing rates of 30–50%, as a result of reactivation of endogenous latent viruses [23,31].

In contrast, recently published results of multi-center studies carried out in a large group (N=298) of solid organ transplant recipients monitored in connection with anti-CMV treatment demonstrated a low (<10%) frequency of HHV-6 and HHV-7 infections, as well as rare instances (<15%) of mixed infections [11]. The authors did not observe co-infections to be associated with a higher degree of immunosuppression and poorer response to CMV infection treatment, nor with the number of recurrent disease episodes. They based their results on examinations of samples collected at the onset of anti-CMV therapy and subsequently on days 3, 7, 14 and 21 of the treatment. In our study, carried out as long as 35 months post-transplant, an important observation is that CMV presence was often preceded by another herpesvirus infection (especially HHV-6). In 18 of 32 patients (56%) with active CMV replication in whom mixed infections were detected, the presence of another herpesvirus preceded the appearance of CMV (Table 4). Observations presented in this paper confirm that the majority of patients infected with 2 or more viruses had sequential and not concurrent infections. Co-infections were relatively rare, only in 15% of samples. The most frequent observation was sequential infections; their appearance might promote an increase in CMV replication intensity (e.g. through an additional immunosuppressive effect resulting from a decreased number of T CD4+ lymphocytes in HHV-6 or HHV-7 infections due to their selective tropism or released cytokines) [32].

There are no reports in the available literature where follow-up was as long as in the present study. In most cases observations were carried out in the early post-transplant period, when immunosuppression is most intense, more often up to 12 weeks [28,29,33]. The present paper draws attention to the common appearance of infections not only in the period of hematologic reconstitution and the associated immunosuppression, but also in the late post-transplant period. Recurrent infections by CMV, but also by EBV, HHV-6 and HHV-7, were observed. Such infections may affect the progression of CMV disease. It is too early to assess if the symptoms observed in patients were truly caused by CMV infection or if other herpesviruses could participate in the development of CMV disease. This observation coincided with the data of others who reported similar co-infections in solid organ recipients [22,34,35]. On the other hand, Pasca et al. [36] did not observe any correlation between HHV-6 and HHV-7 with the presence of CMV genome and with CMV-related clinical manifestations. In our study, EBV, HHV-6 and HHV-7, as well as CMV, were detected in PBLs in asymptomatic individual patients. Such an asymptomatic infection was observed in 16 out of 50 (32%) subjects, but only in groups II and III. In turn, all Group I patients presented clinical symptoms. In a few isolated cases, CMV infection was confirmed "*in situ*" in autopsy specimens (Table 5). High aminotransferases, as an index of liver damage, might have been the result of both a viral infection and advanced GvHD [37,38]. In Group I, chronic GvHD was seen in 13 patients and extensive GvHD in 5. Otherwise, it is well-known that CMV alone or in concomitant presence of other herpesviruses may initiate or exacerbate GvH disease [39], hence it is difficult to pinpoint the cause of such high aminotransferases level in our patients. The possible contribution of HHV-7 to the process of transplant rejection has already been indicated by other authors [29]. On the other hand, Chan et al. [40] did not observe, in pediatric allogeneic recipients of HSCT, a significantly higher risk for development of acute GvHD in HHV-7-positive patients. Based on research presented here, the contribution of lymphotropic herpesviruses to infections, particularly HHV-7, in maintaining CMV replication and disease progression, as well as in chronic GvHD progression, may be suggested. The findings are in accordance with results obtained in solid organ transplant recipients, in which allograft rejection was a stronger predictor of late episodes of CMV disease [41]. Our study demonstrates frequent lymphotropic herpesvirus infections in allogeneic HSCT patients with CMV. Their presence could predispose to symptomatic CMV infection and promote GvHD, and should not be omitted in the study of the pathogenesis of CMV disease.

Conclusions

Our results from long-term observation of allo-HSCT adult recipients confirm frequent lymphotropic herpesviruses infections. Their presence could predispose CMV to cause chronic and recurrent infection. Their monitoring, especially in patients after HSCT, should be considered.

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