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An Assessment of the Effect of Human Herpesvirus-6 Replication on Active Cytomegalovirus Infection after Allogeneic Stem Cell Transplantation

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Human herpesvirus-6 (HHV-6) may enhance cytomegalovirus (CMV) replication in allogeneic stem cell transplant (allo-SCT) recipients either through direct or indirect mechanisms. Definitive evidence supporting this hypothesis are lacking. We investigated the effect of HHV-6 replication on active CMV infection in 68 allo-SCT recipients. Analysis of plasma HHV-6 and CMV DNAemia was performed by real-time PCR. Enumeration of pp65 and IE-1 CMV-specific IFNγ $CD8^+$ and $CD4^+$ T cells was performed by intracellular cytokine staining. HHV-6 DNAemia occurred in 39.8% of patients, and was significantly associated with subsequent CMV DNAemia in univariate ($P = .01$), but not in multivariate analysis ($P = .65$). The peak of HHV-6 DNAemia was not predictive of the development of CMV DNAemia. Timing and kinetics of active CMV infection were comparable in patients either with or without a preceding episode of HHV-6 DNAemia. The occurrence of HHV-6 DNAemia had no impact on CMV-specific T cell immunity reconstitution early after transplant. The receipt of a graft from an HLA-mismatched donor was independently associated with HHV-6 ($P = .009$) and CMV reactivation ($P = .04$). The data favor the hypothesis that a state of severe immunosuppression leads to HHV-6 and CMV coactivation, but argue against a role of HHV-6 in predisposing to the development of CMV DNAemia or influencing the course of active CMV infection.

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KEY WORDS: Human herpesvirus 6 (HHV-6), Cytomegalovirus (CMV), CMV replication, CMV immune response, Interaction, Allo-SCT recipient

INTRODUCTION

Active cytomegalovirus (CMV) infection is a frequent event following allogeneic hematopoietic stem cell transplantation (allo-SCT), which causes significant morbidity and mortality [1]. Human herpesvirus 6 (HHV-6) is a member of the beta herpesvirus subfamily whose reactivation has been increasingly related to clinical complications, such as delayed neutrophil and platelet engraftment, interstitial pneumonia, skin rash, severe graft-versus-host disease (GVHD), central nervous system disorders, and overall mortality [2]. Depending on the method used for the surveillance of viral replication and the net state of post-transplant immunosuppresion, 45% to 85%, and 25% to 80% of allo-SCT recipients experience 1 or more episodes of active CMV or HHV-6 infection, respectively, within the first 100 days after transplantation [1,2]. HHV-6 is considered an immunomodulatory and immunosuppressive agent [3], and thereby might increase the risk of active CMV infection and disease in the transplantation setting. Epidemiologic, clinical, and virologic evidence supporting this assumption has been mainly reported in solid organ transplant recipients. It was shown that occurrence of HHV-6 viremia was associated with an increased risk of subsequent development of either CMV syndrome or CMV end-organ disease [4-9]. The pathophysiologic basis of such viral interaction is largely unknown, but it might be related to the ability of HHV-6 to induce the synthesis of proinflammatory cytokines, such as interleukin-1â and tumor necrosis- á, known to trigger CMV reactivation, and to

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suppress T-lymphocyte function [3]. The effect of HHV-6 replication on CMV infection in the allo-SCT setting and the clinical consequences of such interaction, if any, remain imprecisely defined. In a recent report, Wang et al. [10] provided data suggesting that HHV-6 reactivation might suppress the reconstitution of CMV-specific lymphoproliferative responses, thereby promoting CMV replication. The present study was undertaken to determine whether active HHV-6 infection predisposes to CMV reactivation and/or has any influence on the kinetics of CMV replication in the allo-SCT setting. The elucidation of the nature of the pathogenetic interaction between both beta herpesviruses may have important implications in the therapeutic management of CMV infection in recipients of allo-SCT.

PATIENTS AND METHODS

Patients

Patients undergoing allo-SCT between November 2005, and December 2008, at the following hospitals: Hospital Clínico Universitario,Valencia, Hospital La Princesa, Madrid, Hospital Morales Meseguer, Murcia, and Hospital Ramón y Cajal, Madrid, were eligible for inclusion. The study was approved by all ethics committees. All patients gave their informed consent to participate in the study. Sixty-eight allo-SCT recipients were included and followed up until day 100 after transplantation. Relevant demographic and clinical data of these patients are shown in Table 1.

Table 1. Characteristics of Patients

Parameter	Value
Patients	68
Age, years	45 (18–70)
Sex, male/female	37/31
Underlying disease	
Acute myelogenous leukemia	25 (36.7)
Acute lymphoblastic leukemia	10 (14.7)
Non-Hodgkin lymphoma	8 (11.8)
Hodgkin lymphoma	5 (7.4)
Plasma cell disorders	4 (5.9)
Chronic lymphocytic leukemia	4 (5.9)
Chronic myelogenous leukemia	3 (4.4)
Myelodysplastic syndrome	2 (2.9)
Severe aplastic anemia	1 (1.5)
Others	6 (8.8)
Cytomegalovirus serostatus	
D+/R +	39 (57.3)
D–/R+	21 (30.8)
D–/R–	6 (8.8)
D+/R–	2 (2.9)
Donor type	
HLA-identical sibling	30 (44.1)
Mismatched related donor	6 (8.8)
Matched unrelated donor	19 (27.9)
Mismatched unrelated donor	13 (21.6)
Conditioning regimen	
Nonmyeloablative	40 (58.8)
Myeloablative	28 (41.2)
Stem cell source	
Peripheral blood	53 (77.9)
Umbilical cord blood	13 (19.11)
Bone marrow	2 (2.9)
GvHD prophylaxis	
Cyclosporine A + methotrexate	33 (48.5)
Cyclosporine A + MMF	15 (22.0)
Cyclosporine A + prednisone	14 (20.5)
Others	6 (8.8)
Acute GVHD incidence*	
Grades 0–I	45 (66.1)
Grades II–IV	23 (33.9)

GVHD indicates graft-versus-host disease; MMF, mycophenolate mofetil; D, donor; R, recipient; +, CMV seropositive; –, CMV seronegative; HHV-6, human herpesvirus-6.
Age is given as median (range).
*Incidence of acute GVHD by day +100 posttransplant.

Definitions

Neutrophil and platelet engraftment were defined as: >500 cells/μL for 3 days, and >50,000 platelets/μL for 1 week in the absence of platelet transfusion, respectively, counting the first day as the day of engraftment [11]. The intensity of acute GVHD (aGVHD) was graded from 0 to IV (0 to I, low grade, and II to IV, high grade), as previously described [11].

Virological Monitoring and Management of Active CMV Infection

Patients were monitored for pp65 antigenemia by a commercial immunofluorescence assay (Light Diagnostics® CMV pp65 Antigenemia Immunofluorescence assay, Chemicon International, Temecula, CA, USA), and plasma DNAemia (CMV real-time PCR, Abbott Molecular, Des Plaines, IL, USA) once a week, as previously reported [12,13]. The surveillance of HHV-6 DNAemia was not routinely performed in our patients. Thus, cryopreserved specimens were used for analysis. The availability of a sufficient volume of plasma for PCR was a limiting factor that precluded the analysis of a larger number of specimens. Sequential plasma samples from patients had been collected from a median of 7 days (range: 0-26 days) to a median of 58 days (range: 38-75 days) after transplant. A total of 520 samples (a median of 6 samples per patient; range: 3-11 samples) were analyzed. HHV-6 DNA detection was performed by a commercial real-time PCR assay (HHV-6 Q-PCR Alert Amplimix, Nanogen Advanced Diagnostics, Torino, Italy) on a Smart Cycler instrument (Cepheid, Sunnyvale, CA, USA). DNA was extracted from a volume of 200 μL of plasma on the BioRobot EZ1 workstation (EZ1 virus Mini Kit v2.0, Qiagen, Hamburg, Germany). This assay amplifies a sequence within ORF 13R common to HHV-6 variants A and B. The lower limit of detection of the assay is 10 copies/mL, and the linear measuring range is 6 $\log_{10}$ copies/mL. For data analysis, the commencement and end of a given episode of active viral infection were defined by the first positive (any level of plasma viral load) and first negative results, respectively, in the corresponding PCR assay.

Active HHV-6 or CMV infection was defined by the detection of viral DNA in 1 or more plasma specimens. Preemptive therapy with oral valganciclovir (900 mg/12 hours) or i.v. ganciclovir (5 mg/lg/12 hours) was initiated upon a positive antigenemia result (≥1 pp65 positive cells/200,000 cells) and discontinued following 2 consecutive negative antigenemia results obtained 3 to 7 days apart, after a minimum of 2 weeks of treatment. Foscarnet (i.v. 60 mg/kg/12 hours) was used instead of ganciclovir in patients with severe neutropenia. All patients with antibodies against herpes simplex virus received oral acyclovir prophylaxis following the institutional protocols.

Viral Doubling Time Calculation

To determine the rate of virus replication, the viral doubling time (*dt*) of HHV-6 and CMV was estimated following 2 approaches. In the first, we considered for analysis the first 2 PCR-positive results. The dt was given by $dt = (t2 - t1) \times \log(2)/\log(q2/q1)$, with q1 and t1 being the DNAemia level (copies/mL) at the time of the first positive PCR (in days) respectively, and q2 and t2 the DNAemia level at the time of the second measurement, respectively. This formula assumes a constant growth rate, which occurs in the early phase of virus replication. When more than 2 positive PCR results were available, the peak DNAemia value was not considered, as the growth rate of a virus slows as the viral load approaches its peak. In the second approach, we included in the analysis all measurements from the last negative PCR result to the peak viral load, and estimated the *dt* as $dt = \ln(2)/k$, with k being the rate constant for exponential growth, which is given by $V(t) = V(0)xe^{kt}$, where V is the viral load and t the time (in days) [14]. For CMV, the *dt* was calculated on the basis of DNAemia values obtained prior to initiation of preemptive therapy. Thus, only episodes in which the PCR turned positive (at least 2 consecutive samples) earlier than the antigenemia assay and those resolving without the implementation of preemptive therapy (antigenemia negative episodes) were taken into consideration for analysis. Likewise, for calculation of the HHV-6 *dt* only viral loads measured in the absence of (val)ganciclovir therapy were taken into consideration for analysis.

Immunological Monitoring

Enumeration of pp65 and IE-1 CMV-specific IFNγ-producing $CD8^+$ and $CD4^+$ T lymphocytes was carried out by flow cytometry for ICS (BD Fastimmune, BD-Beckton Dickinson and Company-Biosciences, San Jose, CA, USA) as described previously [15]. Whole blood was simultaneously stimulated with 2 sets of 15-mer overlapping peptides encompassing the sequence of pp65 and IE-1 CMV proteins (2 μg/mL/peptide), obtained from JPT peptide Technologies GmbH (Berlin, Germany), in the presence of 1 μg/mL of costimulatory monoclonal antibodies (mAbs) to CD28 and CD49d for 6 hours at 37° C. Brefeldin A (10 μg/mL) was added for the last 4 hours of incubation. Cells were permeabilized and stained with a combination of labeled mAbs (anti-IFNγ-FITC, anti-CD69-PE, anti-CD4, or CD8-PerCP-Cy5.5 and anti-CD3-APC when the IFNγ $CD8^+$ kit was used). Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences Immunocytometry Systems). $CD4^+$ and $CD8^+$ events were gated and then analyzed for the CD69 activation marker and IFNγ production. The total number of CMV-specific $CD4^+$ and $CD8^+$ T cells was calculated by multiplying the percentages of CMV-specific T cells producing IFNγ upon stimulation (after background substraction) by the absolute $CD4^+$ and $CD8^+$ T cell counts. The specific responses were considered those >0.1% for both $CD4^+$ and $CD8^+$ T cells.

Statistical Analysis

Data were analyzed with the aid of the statistical package SPSS (version 15.0). Frequency comparisons were carried out using the χ^2 test for categoric variables (univariate analysis), and the nonparametric Mann-Whitney *U* test for unpaired continuous data. The Spearman rank test was used for analyzing the correlation between continuous variables. For multivariate analysis, variables associated ($P < .10$) with HHV-6 DNAemia in univariate analysis were included and analyzed using a binary logistic regression model. Variables that were associated with CMV DNAemia in the univariate analysis were included and analyzed using a Cox proportional hazards regression modeling, in which HHV-6 DNAemia was entered as a time-dependent variable. The potential associations between HHV-6 DNAemia and neutrophil and platelet engraftment, and between HHV-6 or CMV DNAemia and the incidence of high grade (II-IV) aGVHD were also evaluated by Cox proportional hazards regression analysis, both virologic events being considered as a time-dependent variables. Results are expressed as relative risk ratios (RRs) or hazard ratios (HRs), and their corresponding 95% confidence interval (CI). A *P* value <.05 was considered significant.

RESULTS

Incidences and Kinetics of Active HHV-6 Infection

Sixty-eight nonconsecutive patients at the Hospital Clínico Universitario, Valencia (n = 53), and the Hospital de la Princesa, Madrid (n = 15) were finally included in the study. In 62 of the 68 patients, the first

specimen subject to analysis was obtained within the first 10 days after transplant. HHV-6 DNAemia was detected at least once in 27 of 68 patients (39.7%), a median of 20 days (range: 7- 44 days) after transplantation. A median of 2 positive samples (range: 1-5 samples) per patient was found. Six patients had a single positive PCR result. To rule out the possibility of DNA contamination, a second aliquot of plasma from these 6 patients was analyzed. All these samples tested positive again and displayed comparable HHV-6 DNA loads (not shown).

The kinetics of HHV-6 DNAemia in our patients is shown in Figure 1. The peak level of HHV-6 DNAemia (median 345 copies/mL, range: 26-13,661 copies/mL) was reached more frequently in the fourth week (52%). Episodes of HHV-6 DNAemia lasted a median of 10 days (range: 3-35 days). Self-clearance of HHV-6 DNAemia was observed in 19 of 27 episodes. Resolution of the remaining 8 episodes of HHV-6 DNAemia occurred while patients were on (val)ganciclovir therapy, which was initiated because of the development of antigenemia-positive active CMV infection. In 3 of these 8 episodes, preemptive therapy was initiated at the time of peak HHV-6 DNAemia. In the remaining 5 episodes, preemptive therapy was implemented once HHV-6 DNAemia had begun to decrease.

Relationship between HHV-6 and CMV DNAemia

Thirty-nine patients (57.3%) experienced an episode of active CMV infection within the first 100 days after transplant (median onset of the episodes 34 days, range: 0-70 days). None of these patients progressed to CMV disease within the study period. Twenty-seven of the 39 episodes were preemptively treated, as the antigenemia assay turned positive. The remaining 12 episodes resolved spontaneously. No PCR negative/antigenemia positive episodes were observed. In all episodes, the plasma PCR assay turned positive earlier than the antigenemia assay.

Twenty-one of the 39 patients had prior or concomitant HHV-6 DNAemia. In 20 of 21 patients, detection of HHV-6 DNAemia preceded that of CMV DNAemia by a median of 15 days (range: 2-50 days). The remaining 18 patients developing CMV DNAemia had no HHV-6 DNA detected in plasma. HHV-6 and CMV DNAemia overlapped in 11 of the 21 patients. In the remaining 10 patients, CMV DNAemia was detected after HHV-6 DNAemia clearance.

The peak level of HHV-6 DNAemia in patients who subsequently experienced an episode of active CMV infection (median 190 copies/mL, range: 26-13,661 copies/mL), and that in patients who did not develop it (median 214 copies/mL, range: 65-478 copies/mL) were not significantly different ($P = .85$). The kinetics profile of CMV DNAemia in patients

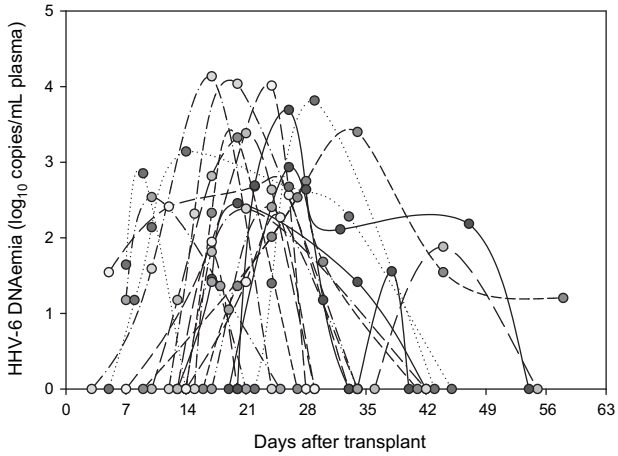

Figure 1. Kinetics of human herpesvirus 6 (HHV-6) plasma DNAemia in 27 allogeneic stem cell transplant recipients who had 1 or more positive results by a real-time PCR (lower detection limit, 10 DNA copies/mL).

with or without a preceding episode of HHV-6 DNAemia was next investigated. As shown in Table 2, we found no significant differences between the groups in terms of the time to the first CMV PCR-positive result, the day of initiation of preemptive treatment and its duration, the initial antigenemia and DNAemia values, the peak viral load, the duration of DNAemia, and the number of preemptively treated or self-resolving episodes. Furthermore, no significant correlation ($P = .43$) was found between the peak levels of HHV-6 and CMV DNAemia.

Doubling Times of HHV-6 and CMV

As a complementary approach to assess the effect of active HHV-6 infection on CMV replication, we estimated the rate of CMV replication in patients with or without a preceding episode of HHV-6 DNAemia. As explained in the methods section, we used 2 different approaches and obtained comparable results (not shown). Data are depicted in Figure 2. The median *dt* of HHV-6 was 0.90 days, ranging from 0.2 to 1.71 days, which was significantly ($P \leq .001$) shorter than that of CMV (median 1.72 days, range: 0.60-12.8 days). The median *dt* of CMV in patients with prior HHV-6 DNAemia was 1.72 days (range: 0.90-12.8 days), and did not differ significantly ($P = .64$) from that in patients with no documented HHV-6 DNAemia (median 1.56 days, range: 1.07-8.50 days). The latter 2 subgroups of patients were matched for donor CMV serostatus, donor type (related/unrelated; matched/mismatched), source of stem cells and conditioning, and GVHD prophylaxis regimens (not shown).

Effect of Active HHV-6 Infection on CMV-Specific Immune Reconstitution Early after Transplant

We investigated whether the occurrence of active HHV-6 infection had any measurable effect on the

Table 2. Active CMV Infection Profile in Patients with or without a Preceding Episode of Human Herpesvirus 6 (HHV-6) DNAemia

Virologic Parameter	HHV-6 DNAemia		*P* Value*
	Yes (n = 21)	No (n = 18)	
First CMV PCR			
Positive, day	29 (6-66)	36 (0-70)	.95
Initial CMV load in copies/mL	41 (25-2300)	45 (25-7276)	.71
Peak CMV load in copies/mL	1207 (25-212,320)	1302 (25-65,945)	.90
Duration CMV			
DNAemia, days	29 (5-100)	24 (3-98)	.91
Initiation of preemptive therapy, days	36 (24-67)	36.5 (12-96)	.21
Initial antigenemia value (positive cells/200,000 PMNLs)	3 (1-210)	3 (1-50)	.46
Self-resolving episodes†	5	6	.51
Duration of preemptive treatment	22 (14-60)	26.5 (14-90)	.71

CMV indicates cytomegalovirus; PCR, polymerase chain reaction; PMNL, polymorphonuclear leukocytes.
Figures are given as median numbers (range).
*Analysis performed by use of Mann-Whitney *U*-test.
†Episodes of CMV DNAemia resolved without implementation of preemptive therapy.

recovery of pp65 and IE-1-specific IFNγ-CD8$^+$ and IFNγ CD4$^+$ T cell responses early after transplant (median 32 days; range: 26–48 days). As shown in Table 3, overall, peripheral counts of both T cell subsets in patients experiencing HHV-6 DNAemia prior to day 30 were not significantly different from those in patients with no documented HHV-6 DNAemia. We performed a similar analysis on 2 subgroups of patients: 1 including patients not developing active CMV infection during the follow-up period (n = 23), of whom 6 had HHV-6 DNAemia and 17 did not, and the other including patients who developed an active CMV infection beyond day 30 posttransplant (n = 14), of whom 6 had a preceding episode of HHV-6 DNAemia and 8 did not. Again, we found comparable levels of both CMV-specific T cell subsets in both subgroups, irrespective of whether a preceding episode of HHV-6 DNAemia had been documented or not.

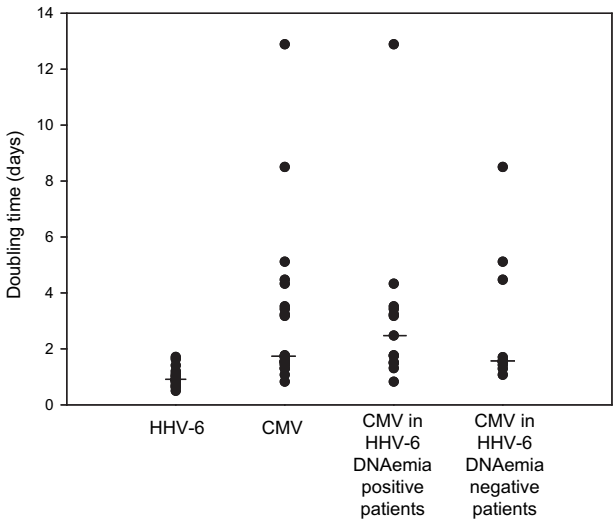

Figure 2. Doubling times (*dt*) in days of human herpes virus 6 (HHV-6) and cytomegalovirus (CMV). The *dt*s of CMV in patients with a preceding episode of HHV-6 DNAemia and in those with no documented HHV-6 DNAemia are represented separately in the right columns. Horizontal bars represent median values.

We also assessed whether occurrence of HHV-6 DNAemia had any effect on the magnitude of the CMV-specific immune response elicited by CMV replication. Blood samples drawn at a median of 63 days (range: 56-74 days) after transplant were available from 14 patients developing active CMV infection within 30 to 60 days after transplant. Seven of these patients had an episode of HHV-6 DNAemia preceding that of active CMV infection. Median IFNγ-CD8$^+$ and IFNγ CD4$^+$ T cells in these patients (0.44 cells/μL, and 0.34 cells/μL, respectively) did not differ significantly ($P = .23$) from that in patients with no prior episode of HHV-6 DNAemia (0.28 cells/μL, and 0.24 cells/μL).

Risk Factors for HHV-6 and CMV DNAemia

In our cohort, receipt of a graft from an unrelated or and HLA-mismatched donor, the use of umbilical cord blood as a source of hematopoietic stem cells, myeloablative conditioning, and the use of prednisone in GVHD prophylaxis were significantly associated with the occurrence of HHV-6 DNAemia in the univariate analysis (Table 4). Other covariates such as age, sex, underlying disease, and donor CMV serostatus were not associated with HHV-6 DNAemia (not shown). In the multivariate analysis, the source of stem cells and the receipt of a graft from an unrelated or an HLA-mismatched donor were independently associated with HHV-6 DNAemia (Table 4).

The univariate analysis identified several risk factors significantly associated with the occurrence of CMV DNAemia: receipt of a graft from an HLA-mismatched donor (RR = 1.5, 95% CI, 1.1-2.4, $P = .04$) or an unrelated donor (RR = 1.6, 95% CI, 1.0-2.4, $P = .01$), use of prednisone in GVHD prophylaxis regimen (RR = 1.8, 95% CI, 0.6-5.0, $P = .00$), and prior HHV-6 DNAemia (RR = 1.6, 95% CI, 1.1-2.4, $P = .01$). Nevertheless, in

Table 3. Early Reconstitution (day +30) of pp65 and IE-1-Specific IFNγ-Producing $CD8^+$ and $CD4^+$T Cells in Patients Either Experiencing or Not human Herpesvirus 6 (HHV-6) DNAemia

Patient Group	HHV-6 DNAemia	No HHV-6 DNAemia	*P* Value*
All patients†			
IFNγ $CD8^+$	0.30 (0-1.54)	0.11 (0-3.11)	.52
IFNγ $CD4^+$	0.32 (0-1.54)	0.18 (0-7)	.54
No CMV DNAemia prior to day +100‡			
IFNγ $CD8^+$	0.92 (0-1.54)	0.42 (0-3.11)	.83
IFNγ $CD4^+$	0.78 (0-3.76)	0.59 (0-7)	.66
CMV DNAemia beyond day +30§			
IFNγ $CD8^+$	0.26 (0-0.56)	0 (0-0.42)	.18
IFNγ $CD4^+$	0.20 (0-1.02)	0.04 (0-0.32)	.34

CMV indicates cytomegalovirus.
Figures are cells/μL blood, and are given as median numbers (range).
*Analysis performed by use of Mann-Whitney *U*-test.
†All 37 patients either experiencing or not CMV DNAemia.
‡This group included 23 patients, of whom 17 developed HHV-6 DNAemia prior to day 30 posttransplant and 6 did not.
§This group included 14 patients, of whom 6 developed HHV-6 DNAemia prior to day 30 posttransplant and 8 did not.

the multivariate analysis, in which HHV-6 DNAemia was introduced as a time-dependent variable, only the receipt of a graft from an HLA-mismatched donor retained significance (Table 5).

HHV-6 DNAemia, CMV DNAemia, and Clinical End Points

We investigated whether HHV-6 DNAemia had any effect on the time to neutrophil and platelet engraftment. All but 1 patient had achieved neutrophil engraftment by day +30, whereas 80% and 92% of patients had achieved platelet engraftment by days +30 and +50, respectively. Only patients displaying HHV-6 DNAemia before day +30 (25 of 27 patients) were included for analysis. The occurrence of HHV-6 DNAemia had no significant effect on neutrophil engraftement by day +30 (HR, 1.0; 95% CI, 0.8-1.7; $P = .85$), or platelet engraftment by day +30 (HR, 0.9; 95% CI, 0.7-2.1; $P = .36$) or by day +50 (HR, 1.1; 95% CI, 0.6-2.7; $P = .54$).

We also assessed the potential effect of HHV-6 and CMV DNAemia in the development of high grade (II to IV) aGVHD by day +100 after transplantation. Both virologic events were considered for analysis only if they preceded the occurrence of the clinical end point. Grades II-IV aGVHD occurred in 7 of 24 patients with no evidence of HHV-6 and CMV DNAemia, and in 16 out of 44 patients who developed either HHV-6 DNAemia, CMV DNAemia, or both. Neither HHV-6 DNAemia (HR, 0.9; 95% CI, 0.3-1.9; $P = .6$) nor CMV DNAemia (HR, 0.8; 95% CI, 0.2-2.1) were significantly associated with subsequent development of high grade aGVHD.

Table 4. Analysis of Risk Factors Associated with the Development of Human Herpesvirus 6 (HHV-6) DNAemia

Risk Factor	RR (95% CI) *P* Value			
	Univariate		Multivariate	
Unrelated donor	4.5 (1.9-10.7)	.00	1.7 (0.4-3.1)	.01
HLA mismatched donor	2.5 (0.8-7.4)	.00	1.8 (0.7-3.8)	.00
Myeloablative conditioning	2.4 (1.3-4.4)	.03	1.2 (0.5-1.4)	.08
Cord blood graft	3.3 (2.1-5.3)	.00	1.8 (0.4-3.3)	.00
Prednisone in GVHD prophylaxis	1.5 (0.5-4.3)	.00	1.2 (0.6-1.9)	.21

RR indicates relative risk ratio (95% confidence interval); GVHD, graft-versus-host disease.

No clinical events potentially associated with HHV-6 reactivation in allo-SCT recipients (CNS dysfunction, idiopathic pneumonia syndrome, or skin rash not because of GVHD) were observed in our cohort. In addition, we sought to determine whether the occurrence of HHV-6 DNAemia, CMV DNAemia, or both had any influence on all-cause mortality during the study period. Eleven patients had died by +100 (5 because of severe aGVHD, 4 because of relapse, and 2 as a result of a systemic infection). Of the 11 patients, 4 had no documented HHV-6 or CMV DNAemia, 5 had only CMV DNAemia, and 2 displayed HHV-6 and CMV DNAemia. The scarce number of clinical events precluded any meaningful statistical analysis.

DISCUSSION

In our cohort of allo-SCT recipients, almost 40% of patients experienced HHV-6 DNAemia, which occurred most frequently around the time of engraftment. This percentage might have been even higher as we could not rule out the occurrence of HHV-6 DNAemia very early after transplant in a couple of patients from whom no plasma samples drawn within the first 4 weeks after transplantation were available for analysis. Incidence rates of active HHV-6 infection ranging from 28% to 78% have been published [11,16-28]. Discrepancies among these studies are likely due to variations in the laboratory method and type of specimen employed for detecting HHV-6, the frequency of virologic monitoring and the clinical characteristics of the patients. We used a highly sensitive real-time PCR assay and plasma instead of leukocytes for quantification of HHV-6 DNAemia. Although it remains a matter of debate [25,29], the level of plasma HHV-6 DNAemia appears to reliably reflect the degree of viral systemic replication. In our cohort, HHV-6 variant B was implicated in all episodes (n = 16) analyzed (not shown), and the kinetics of DNAemia was not suggestive of HHV-6 genome integration [30].

In accordance with previous reports [19,21,25,31], the receipt of a graft from an unrelated or HLA-mismatched donor and the use of umbilical cord blood as

Table 5. HHV-6 DNAemia as a Risk Factor for the Development of Cytomegalovirus (CMV) DNAemia

Risk Factor	HR (95% CI)	*P* Value
Unrelated donor	1.8 (0.7-4.6)	.18
HLA mismatched donor	4.7 (1.1-21.5)	.04
Prednisone in GVHD prophylaxis	1.2 (0.7-1.9)	.40
HHV-6 DNAemia	1.2 (0.5-2.7)	.65

HR indicates hazard ratio (95% confidence interval [CI]); GVHD, graft-versus-host disease.
Analysis was performed by Cox proportional hazards regression. HHV-6 DNAemia was included as a time-dependent variable.

the source of stem cells were independently associated with the development of HHV-6 DNAemia.

The kinetics of HHV-6 DNAemia in our patients was comparable with that reported in other series [11,19-22,24-27]. That is, peak incidences occurring within the second and third weeks after transplant, peak viral load being reached within 3 to 4 weeks post-transplant, and median duration of episodes <2 weeks. In our setting, most episodes were self-limited, that is, they resolved without the implementation of antivirals with activity against HHV-6, such as (val)ganciclovir. This appears to be the most frequent outcome of active HHV-6 infection in patients with low to intermediate levels of HHV-6 DNAemia [32], as those in our series. The peak plasma levels of HHV-6 DNAemia in our patients were comparable with those reported by Zerr et al. [11], but markedly lower than those published by Ogata et al. [25]. Again, differences in the characteristics of patients and/or in the method used for HHV-6 DNA quantification may account for this discrepancy.

Contradictory data have been reported on the influence of HHV-6 DNAemia on the time to neutrophil and platelet engraftment [11,19,25,26]. In our cohort, we observed no effect of HHV-6 DNAemia in neutrophil engraftment by day +30, and platelet engraftment by days +30 and +50. The limited number of events included in our analysis does not allow us to draw definitive conclusions on this matter. We also found no significant association between the occurrence of HHV-6 or CMV DNAemia and an increased risk of development of high grade (II to IV) aGVHD. This association has been previously documented for HHV-6 [11,33], but not for CMV [33]. Again, the scarce number of clinical events included minimizes the significance of our analysis.

HHV-6 is known to display immunomodulating and immunosuppressive properties, and thereby it may lead to enhanced CMV replication [4]. In this context, HHV-6 replication has been related to an increased risk of active CMV infection and disease in solid organ transplant recipients [4-9]. Nevertheless, little is known about the biologic and clinical consequences of the interaction between both beta herpesviruses in the allo-SCT setting. Should the role of HHV-6 in promoting CMV replication be proven, then the documentation of HHV-6 DNAemia could become an indication for prophylaxis against CMV infection to prevent or minimize the effects of such an interaction. Previous studies showed that allo-SCT recipients experiencing HHV-6 DNAemia are more prone to subsequently developing an episode of active CMV infection, as determined by blood culture, pp65 antigenemia or leukocytes or plasma CMV DNAemia [10,19,23,24,25,26,28], although this association did not reach significance in all series. Yet, there is no evidence of a relationship between active HHV-6 infection and a higher risk for CMV disease. In our series, an association between HHV-6 DNAemia and the subsequent occurrence of CMV DNAemia was observed, yet most episodes of HHV-6 DNAemia had been cleared spontaneously or were in the process of resolution at the time of CMV DNAemia detection. In fact, overlapping HHV-6 and CMV DNAemia was only observed in around half of patients. Interestingly, the peak level of HHV-6 DNAemia in patients who subsequently developed an episode of CMV DNAemia was comparable with that in patients who did not. Although this observation argues against a direct role of HHV-6 in predisposing to the development of active CMV infection, the possibility that active HHV-6 infection triggered CMV reactivation by a direct interaction independent of the viral load could not be definitely rule out.

We were interested in determining whether the occurrence of HHV-6 DNAemia had any impact on the pattern of active CMV infection. Our data do not support this hypothesis. First, no correlation was found between the peak levels of HHV-6 and CMV DNAemia. Such a correlation would be expected in a scenario where HHV-6 directly interacts with CMV [9]. Our observation is in keeping with a previous report [19], but in contrast to that of others [26]. In the latter study, however, active CMV infection was monitored by CMV antigenemia assay. Second, the occurrence of HHV-6 DNAemia had no apparent impact on the kinetics of active CMV infection, as assessed by the precocity of onset, initial, and peak levels of CMV DNAemia and the viral doubling time. Furthermore, the duration of CMV DNAemia was comparable in patients with or without a preceding episode of HHV-6 DNAemia. This observation was not biased by a different use of val(ganciclovir) in either group, as the number of episodes preemptively treated and the duration of antiviral courses were not significantly different between groups.

Our data are also against a role of HHV-6 in promoting CMV replication by inhibiting the reconstitution of CMV-specific T cell immunity. In a previous study, a trend for an inverse correlation between HHV-6 viral load and the presence of CMV-specific

lymphoproliferative responses was reported [10]. We previously showed that peripheral blood levels of pp65 and IE-1 CMV-specific IFNγ-producing $CD8^+$ and $CD4^+$ T cells correlate with protection against CMV DNAemia [15]. We measured peripheral counts of both T cell subsets around day 30 posttransplant in patients either experiencing an episode of HHV-6 DNAemia prior to that time point or not developing it during the study period. We observed no apparent effect of HHV-6 DNAemia on the level of reconstitution of either T cell population, regardless of whether CMV DNAemia subsequently developed or not. As expected on the basis of previous investigations of our group [15], lower levels of CMV-specific T cells were seen in patients experiencing CMV DNAemia than in those who remained free of it. Differences in the type of specimen used for HHV-6 DNA detection, the method for assessing the immune response against CMV, or the timing of immunologic monitoring may account for the discrepancy between our study and that of Wang et al. [10]. We also observed that peripheral levels of both T cell subsets by day +60 in patients developing an episode of active CMV infection within 30 to 60 days after transplant were comparable in patients with or without a preceding episode of HHV-6 DNAemia. Taken together, the data indicated that occurrence of HHV-6 DNAemia had no effect either in the reconstitution of CMV-specific immunity or in the magnitude of the CMV-specific immune response elicited by CMV replication. It remains to be determined whether the occurrence of HHV-6 DNAemia has an effect on recovery of CMV-specific immunity at later times after transplant.

The temporal association between HHV-6 and CMV DNAemia may just be the result of a severe immunosuppressive condition of patients that triggers viral coactivation, and not necessarily because of their interaction. In line with this assumption, we found the receipt of a graft from an HLA-mismatched donor being independently associated with the occurrence of either HHV-6 or CMV DNAemia in our cohort. Moreover, in the multivariate model, including risk factors that were associated significantly with the development of CMV DNAemia in the univariate analysis, HHV-6 DNAemia did not retain significance, although a trend for an association was evident.

The earlier appearance of HHV-6 in the blood compartment with respect to that of CMV does not necessarily imply a different temporal pattern of reactivation. In fact, precise dating of beta herpesvirus reactivations may only be achieved by virologic monitoring at mucosal or tissue sites [34]. The rate of virus replication may be a relevant factor in determining the precocity of the onset of viral DNAemia. In this context, we found the doubling time of HHV-6 was around half that of CMV (0.9 versus 1.7 days), which is in accordance with a previous estimation [35]. Other pathogenetic factors, such as the ease of access into the blood compartment and the relative stringency of virus-cell association, which may differ between both beta herpesviruses, might also be of relevance.

In conclusion, our data favor the hypothesis that a state of severe immunosuppression leads to HHV-6 and CMV coactivation, but argue against a role of HHV-6 in predisposing the development of CMV DNAemia or influencing the course of active CMV infection. Nevertheless, validation of this assumption requires further studies analyzing larger series of patients.

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