Bone Marrow Failure Associated with Herpesvirus 8 Infection in a Patient Undergoing Autologous Peripheral Blood Stem Cell Transplantation

Maria Cuzzola, Giuseppe Irrera, Olga Iacopino, Angela Cuzzocrea, Giuseppe Messina, Giuseppe Console, Pasquale Iacopino, and Fortunato Morabito

Bone Marrow Transplant Unit, Azienda Ospedaliera Bianchi-Melacrino-Morelli, Reggio Calabria, Italy

We describe a fatal case of human herpesvirus 8–associated bone marrow failure in a patient who had received intense treatment for Hodgkin lymphoma and was undergoing bone marrow transplantation. Bone marrow failure was resistant to antiviral treatment and a second infusion of autologous stem cells. Human herpesvirus 8 infection continues to be a major concern in transplant recipients in critical condition.

It is well known that iatrogenic immunosuppression leads to reactivation of latent viral infections, such as human cytomegalovirus (hCMV), Epstein-Barr virus (EBV), and human herpesvirus 6 (HHV-6) infections [1]. More recently, Kaposi sarcoma–associated herpesvirus, also known as "human herpesvirus type 8," has been identified [2]. HHV-8 is not ubiquitous in the general population; seroprevalence rates are very low in the United Kingdom and the United States and are higher only in certain geographic areas of the world (the Mediterranean area and Africa) [3]. In one study, 17.7% and 18.7% of Italian blood donors had antibodies to latency-associated nuclear antigen and open-reading frame (ORF) 65 protein, respectively, and 24.1% had antibodies to $\geqslant$1 antigen [4]. Moreover, HHV-8 is frequently secreted in saliva, which suggests that saliva is probably the major transmission vehicle [5]. The increase in the prevalence of HHV-8 seropositivity among persons who have received transplants is considered to be a new problem, caused most probably by transfusion procedures [6]. Because HHV-8 transmission has been reported to result from organ transplantation and intravenous drug use, bone marrow transplant (BMT) recipients could be at risk for HHV-8 infection. However, little is known about risk factors for posttransplantation HHV-8 disease, and the association between HHV-8 and delay in engraftment remains to be elucidated for different types of transplants. To date, only a few reports have been published on microbiologically proven HHV-8 infection in transplant recipients [7]. We describe a case of fatal posttransplantation bone marrow aplasia associated with HHV-8 infection in a patient with Hodgkin lymphoma.

Case report. In March 1998, a 17-year-old girl with a diagnosis of stage III Hodgkin lymphoma, subtype nodular sclerosis, was referred to the Bone Marrow Transplant Center (Azienda Ospedaliera Bianchi-Melacrino-Morelli, Reggio Calabria, Italy). The patient's lymphoma was resistant to 4 cycles of a chemotherapy regimen that included etoposide (60 mg/m^2), vincristine (1 mg/m^2), and epirubicin (25 mg/m^2). Therefore, she received 6 cycles of a regimen that included, sequentially, mechlorethamine (6 mg/m^2), lomustine (100 mg/m^2), vindesine (3 mg/m^2), prednisone (40 mg/m^2), epirubicin (40 mg/m^2), vincristine (1.4 mg/m^2), procarbazine (100 mg/m^2), vinblastine (6 mg/m^2), and bleomycin (10 mg/m^2).

After the second cycle of the second chemotherapy regimen, granulocyte colony–stimulating factor was added, and 7.8×10^6 CD34$^+$ peripheral blood stem cells/kg were collected. Because of pulmonary embolism, anticoagulant therapy was also started. In September 2000, after an initial partial response, the patient experienced disease progression in lymph nodes and the lungs. In addition, examination of a bone marrow biopsy specimen showed disease infiltration.

On the basis of these clinical data, it was decided to begin a third-line chemotherapy regimen that consisted of 2 cycles of doxorubicin (25 mg/m^2), bleomycin (10 mg/m^2), vinblastine (6 mg/m^2), and dacarbazine (375 mg/m^2), followed by high-dose sequential therapy. The patient underwent a second stem cell mobilization, and 7.8×10^6 CD34$^+$ peripheral blood stem cells/kg were collected again. After 3 months, the patient received conditioning therapy with mitoxantrone (60 mg/m^2) and melphalan (140 mg/m^2), followed by autologous peripheral blood stem cell rescue (5.5×10^6 CD34$^+$ cells/kg). Antimicrobial prophylactic treatment with ciprofloxacin (500 mg b.i.d.), itraconazole oral solution (5 mg/kg b.i.d.), and acyclovir (5 mg/kg t.i.d.) was also administered.

Time to neutrophil and platelet engraftment was 9 and 12

Table 1. Viral pathogens detected during the leukopenic phase after peripheral blood stem cell transplantation.

Pathogen, indicator	Fraction type	Result of IFA	Result of PCR
Human cytomegalovirus			
pp65 antigen	Cellular	Negative	…
pp67 antigen and mRNA	Cellular	…	Negative
Human herpesvirus 6			
Antigenemia	Cellular	Negative	…
DNA	Cellular	Negative	Negative
Human herpesvirus 8			
DNA	Cellular and acellular	…	Positive
mRNA	Cellular and acellular	…	Positive

**NOTE.** IFA, immunofluorescence assay.

days, respectively. The patient was discharged on day 12 after transplantation. Soon after, the patient presented with cutaneous rash, fever, and leukopenia. A significant decrease of trilineage bone marrow cellularity, without neoplastic infiltration, was documented. The results of tests for antibodies to hepatitis A, B, and C viruses were negative. The results of tests for CMV, EBV, and HHV-6 antigenemia and for HHV-6 DNA and CMV mRNA were also negative (table 1). However, HHV-8 DNA was clearly documented in the acellular and cellular fractions of peripheral blood and bone marrow (figure 1). In addition, HHV-8 mRNA was detected in bone marrow mononuclear cells (figure 2). Because of the progressive lack of marrow cellularity, 9×10^6 autologous CD34$^+$ cells/kg were infused. In addition, hematopoietic growth factors were administered, along with steroids. Platelet and RBC transfusions were also given. Finally, a different antiviral therapy was attempted (foscarnet, 60 mg/kg b.i.d.).

Despite these treatments, HHV-8 DNA and mRNA continued to be detected in both bone marrow and peripheral blood (data not shown), and no evidence of other associated infections was found. On day 95 after transplantation, cytopenia persisted, and the patient died of acute respiratory distress syndrome.

Materials and methods. Mononuclear cells were isolated from heparinized peripheral blood and bone marrow samples by density gradient centrifugation, using Biocoll Separation Solution (Biochrom-BioDivision). The patient was monitored for hCMV, EBV, and HHV-6 infections by testing for pp65 and for ZEBRA and TAN-HST (both from Bioline Diagnostici) antigenemia, respectively. hCMV antigenemia was determined in pp65-negative polymorphonucleated cells after staining with 3 specific monoclonal antibodies (CMV protein kinase 65 CINA pool, clone IC3+AYM; Bioline Diagnostici), as described elsewhere [8]. Similar procedures were performed to test for EBV and HHV-6 antigenemia in mononuclear cells stained with specific monoclonal antibodies (for EBV [ZEBRA], clone AZ-69, and for HHV-

6 [TAN-SHT], clone 7C7 45/15; Bioline Diagnostici). The Nuclisens hCMV pp67 mRNA assay was performed by qualitative nucleic acid sequence–based amplification, according to the manufacturer's instructions (Organon Teknika) [9]. For detection of HHV-6 and HHV-8 DNA in serum and mononuclear cell samples, DNA was extracted with 2 commercial DNA isolation kits (Micro-Geno DNA and Mini-Geno DNA kits; AB Analitica), resuspended in a 100-mmol/L Tris solution containing 50 mmol/L EDTA (pH 8.0), quantified using a UV spectrophotometer reader (optical density at 260 nm), and stored at −20°C until use.

Ten microliters of DNA was used to amplify ORF U31 of HHV-6 by nested PCR assay and ORF K8.1 of HHV-8 by a single-step PCR assay (2 commercial kits were used: the Ampliquality HHV-6 and Ampliquality HHV-8 kits [AB Analitica]). An HHV-8 DNA fragment size of 527 bp was expected. The quality of all DNA samples was tested by PCR assay of β-
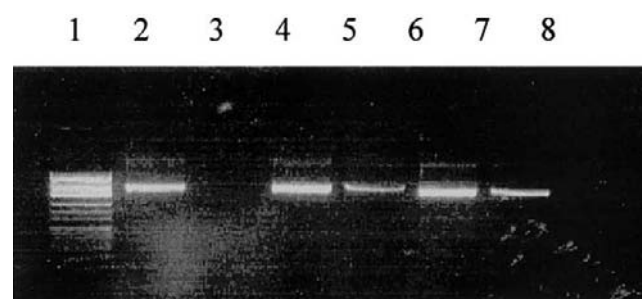
Figure 1. Detection of human herpesvirus 8 DNA in acellular and cellular fractions of peripheral blood or bone marrow. *Lane 1,* molecular weight marker (ϕX174 DNA/Inf I); *lane 2,* PCR analysis of PBMCs positive for open-reading frame (ORF) K8.1 DNA before peripheral blood stem cell transplantation (PBSCT); *lane 3,* serum negative for ORF K8.1 DNA before PBSCT; *lanes 4* and *5,* PBMCs and serum, respectively, both positive for ORF K8.1 DNA after PBSCT; *lanes 6* and *7,* bone marrow mononuclear cells and serum, respectively, positive for ORF K8.1 DNA after PBSCT; *lane 8,* mix PCR without DNA.
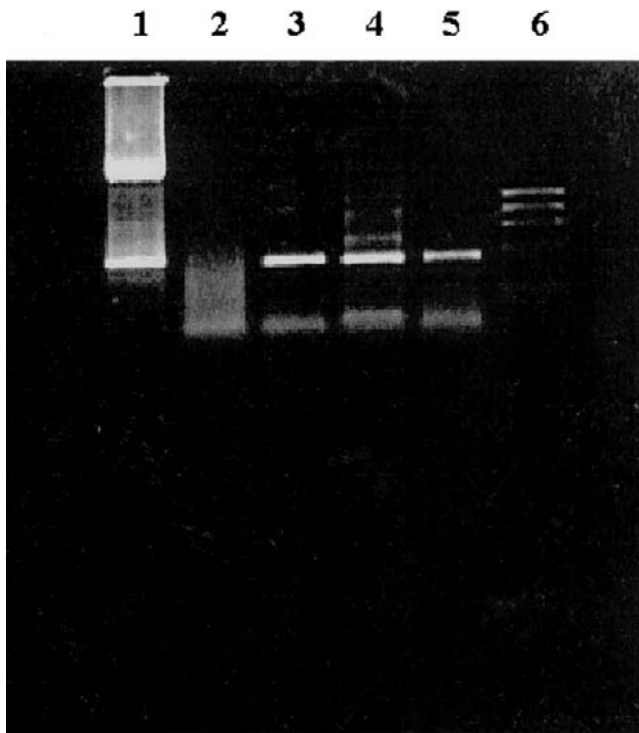
Figure 2. Detection of human herpesvirus 8 DNA in acellular and cellular fractions of peripheral blood or bone marrow. *Lane 1,* RT-PCR analysis of bone marrow mononuclear cells positive for open-reading frame (ORF) K8.1 mRNA after peripheral blood stem cell transplantation (PBSCT); *lane 2,* mix PCR without reverse transcriptase; *lane 3,* serum before peripheral blood stem cell transplantation; *lanes 4* and *5,* PCR analysis of PBMCs and bone marrow mononuclear cells, respectively, both positive for ORF K8.1 DNA after PBSCT (first detection); *lane 6,* molecular weight marker (ϕDNA/Inf I).

globin, and its expected fragment size of 268 bp was used as a control. Assay specificity was confirmed by positive results when HHV-6 and HHV-8 DNA controls were used. Positive controls run with the samples included a negative water blank. PCR products were detected by electrophoresis in 2% agarose gels and visualized after ethidium bromide staining. Molecular weight markers were included in each gel.

HHV-8 K8.1 cDNA was obtained by RT-PCR. mRNA from cells was isolated by using an mRNA isolation kit (Blood RNA kit; AB Analitica). Approximately 0.3 μg of mRNA was reverse transcribed with the Ampliquality HHV-8 kit, according to the manufacturer's instructions. As a negative control, cDNA synthesis was performed without reverse transcriptase. One-microliter aliquots of the same cDNA preparation were used for PCR amplification in 50 μL of final reaction mixture with specific primers designed to amplify 433-bp and 250-bp fragments within ORF K8.1.

Discussion. The occurrence of primary or reactivated human herpesvirus infections among transplant recipients is associated with immunosuppressive therapy and causes serious

and harmful complications. Multiple concomitant viral infections might also enhance the lytic-cycle spread of emerging infectious pathogens, such as HHV-8. This is a γ-herpesvirus that has been shown by genomic sequence analysis to be closely related to herpesvirus saimiri and rhesus monkey rhadinovirus [10]. The biological characteristics of this virus are very interesting for its possible pathogenetic role in neurological and lymphoproliferative diseases [11, 12].

Previous studies have shown that HHV-8 may play a role in supporting bone marrow plasma cellular proliferation and/or neoplastic transformation in patients with multiple myeloma, which suggests that the viral agent may be very important in the development of this B cell malignancy [13]. However, the same microorganism may have a variable pathogenic role in malignant and nonmalignant diseases in human hosts [14]. Primary infection with HHV-8 or HHV-8 reactivation may be associated with different pathologic events that occur after transplantation [15].

HHV-8 infection causes a viruslike syndrome that involves fever, splenomegaly, and myelosuppressive activity in both solid-organ and BMT recipients [7]. In transplant recipients, transient reactivation of HHV-8 is associated with fever, cutaneous rash, and hepatitis. These clinical features are similar to those of infections caused by other members of the herpesvirus family, such as HHV-6 and hCMV. Notably, acute and persistent HHV-8 replication has been associated with high morbidity and mortality.

Figure 3. Detection of human herpesvirus 8 DNA in cellular fractions of peripheral blood and bone marrow before and after peripheral blood stem cell transplantation (PBSCT). *Lane 1,* Low molecular weight marker; *lanes 2* and *3,* PCR analysis of PBMCs positive for open-reading frame (ORF) K8.1 DNA before PBSCT; *lanes 4* and *5,* PCR analysis of PBMCs and bone marrow mononuclear cells, respectively, both positive for ORF K8.1 DNA after PBSCT (first detection); *lanes 6* and *7,* PCR analysis of PBMCs and bone marrow mononuclear cells, respectively, positive for ORF K8.1 DNA after PBSCT (second detection); *lane 8,* mix PCR without DNA.

We attempted to link the clinical and hematological signs in our patient, such as cutaneous rash, fever, and bone marrow suppression, to the results of laboratory and molecular investigation in which tests for several herpesvirus groups were done. Bone marrow, peripheral blood, and serum specimens were serially examined, with the aim of finding evidence of active hCMV, EBV, HHV-6, or HHV-8 replication. Assays for hCMV, EBV, and HHV-6 antigenemia, as well as molecular tests for hCMV mRNA and HHV-6 DNA, had negative results during the symptomatic period. In contrast, HHV-8 DNA was detected by single-step PCR in mononuclear cells in both blood and bone marrow samples. Therefore, to examine the virus life cycle stage, we looked for mRNA of the HHV-8 structural gene transcript K8.1. This was found in all hematological samples during the period after autologous bone marrow transplantation. Because the results of tests for hCMV, EBV, and HHV-6 were negative, we concluded that HHV-8 was the most likely cause of this patient's disorder.

It should be emphasized that evidence needs to be carefully collected before the presence of microbial nucleic acid sequences in blood is definitively correlated with a specific infectious disease. Detection of HHV-8 DNA has been the mainstay for confirming the presence of this virus in tissue. Because nested DNA PCR is particularly prone to contamination and the production of false-positive results, we decided to use a single-step PCR assay to detect HHV-8 DNA. However, PCR detection of specific DNA sequences is not able to distinguish between the latent and the lytic stages of the virus life cycle.

Demonstration of HHV-8 mRNA by RT-PCR, especially of structural gene transcripts, appears to offer the most accurate reflection of viral replication. Detection of structural gene transcripts is indicative of chronic persistent infection. Previous studies have reported that lytic-cycle polypeptides from HHV-8 are predominantly encoded by ORF K8.1, which is present in the same genomic position as virion envelope glycoproteins of other γ-herpesviruses [16]. K8.1 was found to be expressed during lytic HHV-8 replication in body cavity–based lymphoma 1 cells and was localized on the surface of cells and virions [17]. Previous studies have shown that 2 spliced mRNAs are transcribed from the K8.1 locus of HHV-8 (K8.1α and K8.1β), in addition to an unspliced message (K8.1γ) [18, 19]. The primers of the Ampliquality HHV-8 kit recognize a fragment deleted by splicing of the K8.1 locus, and, therefore, a smaller fragment than is expected to result from DNA amplification (latent genome) will be obtained. This procedure ensures the specificity of the amplified fragment, even when viral DNA is present in the RNA sample, which makes quality-control procedures or the use of DNase unnecessary.

In our case, 2 observations suggest that HHV-8 viremia was probably caused by viral reactivation, rather than by primary infection. Both DNA and mRNA were found in sequential samples obtained at the time of clinical symptoms and during the posttransplantation period. On the other hand, only HHV-8 DNA was detected in peripheral blood before transplantation, and we failed to detect any copies of mRNA K8.1. Unfortunately, it was not possible to perform a quantitative molecular kinetic analysis of HHV-8 load, and only qualitative analysis was done (figure 3).

A recent study, in which a real-time PCR assay was used, provided evidence in support of a strong correlation between ingravescent clinical features and a high HHV-8 load [20]. In addition, Quinlivan et al. [21] suggest that HHV-8 copy number, as determined by quantitative-competitive PCR, should be used as biomarker during clinical follow-up. Our report is in agreement with the observations of other groups and confirms the association between HHV-8 and bone marrow failure in patients who have undergone autologous transplantation. The detection of lytic protein transcript mRNA, a method for proving a clinical suspicion, is a clear indicator of chronic and persistent infection; this supports the idea that a strong and close relationship exists between the presence of actively infected cells and adverse outcome in HHV-8–associated disease.

In conclusion, severe manifestation of HHV-8 infection may result in irreversible aplasia in BMT recipients. Therefore, in such subjects, the care usually exercised in dealing with other latent pathogens should be applied to early recognition and appropriate treatment of HHV-8 syndrome. Monitoring the HHV-8 load might be useful as a reference standard for measuring the success of therapy for HHV-8.

References

1. Griffiths PD. Viral complication after transplantation. J Antimicrob Chemother **1995**; 36:91–106.
2. Chang Y, Cesarman E, Pessin MS, et al. Identification of virus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **1994**; 266:1865–9.
3. Gao SJ, Kingsley L, Li M, et al. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. Nat Med **1996**; 2:925–8.
4. Calabro ML, Sheldon J, Favero A, et al. Seroprevalence of Kaposi's sarcoma–associated herpesvirus/human herpesvirus 8 in several regions of Italy. J Hum Virol **1998**; 1:207–13.
5. Blackbourn DJ, Lennette ET, Ambroziak D, Mourich DV, Levy JA. Human herpesvirus 8 detection in nasal secretions and saliva. J Infect Dis **1998**; 177:213–6.
6. Corey L, Brodie S, Huang ML, Koelle DM, Wald A. HHV-8 infection: a model for reactivation and transmission. Rev Med Virol **2002**; 12: 47–63.
7. Luppi M, Barozzi P, Schulz TF, et al. Bone marrow failure associated with human herpesvirus 8 infection after transplantation. N Engl J Med **2000**; 343:1378–85.
8. Boeckh M, Bowden RA, Goodrich JM, Pettinger M, Meyers JD. Cytomegalovirus antigen detection in peripheral blood leukocytes after allogenic marrow transplantation. Blood **1992**; 80:1358–64.
9. Blok MJ, Goossens VJ, Vanherle SJV, et al. Diagnostic value of monitoring human cytomegalovirus late pp67 mRNA expression in renal-

allograft recipients by nucleic acid sequence–based amplification. J Clin Microbiol **1998**; 36:1341–6.

10. Desrosiers RC, Sasseville VG, Czajak SC, et al. A herpesvirus of rhesus monkeys related to human Kaposi sarcoma–associated herpesvirus. J Virol **1997**; 71:9764–9.

11. Sola P, Bedin R, Casoni F, Barozzi P, Mandrioli J, Merelli E. New insights into the viral theory of amyotrophic lateral sclerosis: study on the possible role of Kaposi's sarcoma–associated virus/human herpesvirus 8. Eur Neurol **2002**; 47:108–12.

12. Menke DM, Chadbum A, Cesarman E, et al. Analysis of the human herpesvirus 8 (HHV-8) genome and vIL-6 expression in archival cases of castelman disease at low risk for HIV infection. Am J Clin Pathol **2002**; 117:268–75.

13. Said JW, Rettig MR, Heppner K, et al. Localization of Kaposi's sarcoma–associated herpesvirus in bone marrow biopsy samples from patients with multiple myeloma. Blood **1997**; 90:4278–82.

14. Luppi M, Barozzi P, Schulz TF, et al. Nonmalignant disease associated with human herpesvirus 8 reactivation in patients who have undergone autologous peripheral blood stem cell transplantation. Blood **2000**; 96: 2355–7.

15. Jenkins FJ, Hoffman LJ, Liegey-Dougall L. Reactivation and primary infection with human herpes 8 among solid-organ transplant recipients. J Infect Dis **2002**; 185:1238–41.

16. Li M, MacKey J, Czajak SC, Desrosiers RC, Lackner AA, Jung JU. Identification and characterization of Kaposi's sarcoma–associated herpesvirus K8.1 virion glycoprotein. J Virol **1999**; 73:1341–9.

17. Platt GM, Cannell E, Cuomo ME, Singh S, Mittnacht S. Detection of human herpesvirus 8–encoded cyclin protein in primary effusion lymphoma derived cell-lines. Virology **2000**; 272:257–66.

18. Raab M-S, Albrecht J-C, Birkmann A, et al. The immunogenic glycoprotein gp35–37 of human herpesvirus 8 is encoded by open reading frame K8.1. J Virol **1998**; 72:6725–31.

19. Chandran B, Bloomer C, Chan S-R, Zhu L, Goldstein E, Horvat R. Human herpesvirus-8 ORF K8.1 gene encodes immunogenic glycoproteins generated by spliced transcripts. Virology **1998**; 249:140–9.

20. Lallemand F, Desire N, Rozenbaum W. Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay. J Clin Microbiol **2000**; 38:1404–8.

21. Quinlivan EB, Zhang C, Stewart PW, Komoltri C, Davis MG, Wehbie RS. Elevated virus loads of Kaposi's sarcoma–associated human herpesvirus 8 predict Kaposi's sarcoma disease progression, but elevated levels of human immunodeficiency virus type 1 do not. J Infect Dis **2002**; 185:1736–44.