Human Herpesvirus 6: Infection and Disease Following Autologous and Allogeneic Bone Marrow Transplantation

By Madhavi P. Kadakia, Witold B. Rybka, John A. Stewart, Joanne L. Patton, Felicia R. Starney, Magdy Elsayaw, Philip E. Pellett, and John A. Armstrong

Human herpesvirus 6 activity (HHV-6) was studied in 15 allogeneic and 11 autologous marrow transplantation patients. After transplantation, HHV-6 was isolated from the peripheral blood mononuclear cells of 12 of 26 patients (6 allogeneic and 6 autologous). All isolates were variant B. Eleven of 26 and 12 of 19 patients showed salivary shedding of HHV-6 DNA before and after transplantation, respectively. The antibody titer increased in 7 of 26 patients. Thus, 23 of 26 patients showed evidence of active HHV-6 infection either by virus isolation, salivary shedding, or increases in antibody titers. The fraction of salivary specimens positive in 19 patients was negatively associated with their antibody titers (P = .005). The proportion of cultures positive increased after transplantation (P = .007). Sinusitis was associated with HHV-6 isolation in autologous recipients (P = .002). In allogeneic patients, active human cytomegalovirus infection was associated with HHV-6 isolation (P = .04). No association was observed between HHV-6 infection and GVHD, pneumonia, delay in engraftment, or marrow suppression. Of the 120 clinical events analyzed in 26 patients, HHV-6 was defined as a probable cause of 16 events in 9 patients based on the propinquity of HHV-6 activity and the clinical event plus the absence of other identified causes of the event. This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Patients' Characteristics

All subjects scheduled to undergo BMT at the Pittsburgh Cancer Institute during the study period and their donors were informed of the study. The study protocol and the consent forms were approved by the Biomedical Institutional Review Board of the University of Pittsburgh. All potential candidates consented to be in the study.

Patient characteristics as regards to age, sex, diagnosis, conditioning regimen, type of transplant, duration of follow-up, and the final outcome of the patient are summarized in Table 1. Fifteen of the 26 patients underwent allogeneic BMT and the remaining 11 underwent autologous BMT. Eight of the 15 allogeneic transplant patients received marrow from matched related donors (MRD) and the remaining 7 received marrow from matched unrelated donors (MUD). Six of the 11 autologous transplant patients received BM alone and the remaining 5 received peripheral blood stem cells (PBSC) alone.

Patients were enrolled sequentially in the study as they were admitted to hospital for transplantation to minimize selection bias. The enrollment of patients occurred in two phases. The pilot phase included the first 7 patients enrolled over 3 months. The second phase consisted of 19 additional patients enrolled over 4 months.

Treatment Regimens

The pretransplant conditioning regimen (Table 1) consisted of one of the following regimens: (1) cyclophosphamide (Cy; 120 mg/kg) and total body irradiation (TBI; 1,330 cGy); (2) etoposide (VP16; 60 mg/kg) and TBI (1,320 cGy); (3) Cy (120 mg/kg) and busulfan (Bu; 16 mg/kg); (4) VP16 (1,606 mg/m²), Cy (100 mg/kg), and carboplatin (Cb; 1,500 mg/m²); and (5) Cy (6,000 mg/m²), thiopeta (500 mg/m²), and Cb (800 mg/m²). Allogeneic and autologous BMT recipients received 3 × 10⁹ nucleated marrow cells per kilogram of recipient body weight. PBSC transplant recipients received 7 × 10⁹ mononuclear cells per kilogram of body weight. 20,24 GVHD prophylaxis was given with cyclosporine (CsA) and methotrexate (MTX).

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Experimental Design

Pretransplantation heparinized blood was obtained from allogeneic and autologous donors. In addition, samples of harvested BM used for transplantation were obtained for patients receiving BM and samples of the harvested PBSC used for transplantation were obtained from patients receiving PBSC. Heparinized blood was obtained from all the recipients twice before transplantation as baseline, and samples of the harvested PBSC used for transplantation were obtained for patients receiving BM and autologous donors. In addition, samples of harvested BM used for transplantation were obtained for patients receiving BM and autologous donors. When possible, bronchoalveolar lavage (BAL) specimens were obtained before transplantation from donors and from recipients at all the times when blood was collected from recipients. In the study phase (patients no. 1 through 7), urine and saliva were studied before transplantation. In the pilot study (patients no. 8 through 26), no urine specimens were studied, but saliva specimens were collected before transplantation from donors and from recipients at all the times when blood was collected from recipients. Pretransplantation heparinized blood was obtained from all the recipients twice before transplantation as baseline, weekly for 1 month, and monthly for up to 3 months. In addition, a late follow-up sample was obtained between 6 months and 1 year after transplantation when possible. In the pilot study (patients no. 1 through 7), urine and saliva were studied before transplantation from both the recipients and their donors. In the study phase (patients no. 8 through 26), no urine specimens were studied, but saliva specimens were collected before transplantation from donors and from recipients at all the times when blood was collected from recipients. When possible, bronchoalveolar lavage (BAL) specimens were obtained from recipients on whom BAL was performed when they developed pneumonia. Skin biopsy specimens were obtained if a patient developed skin rash. Clinical data were collected prospectively on all 26 recipients, summarized at the end of the study, and assessed to determine associations with active HHV-6 infection.

Collection and Storage of the Specimens

Heparinized blood was collected and stored on a rocker at room temperature for less than 24 hours. Cells saved for PCR were frozen as cell pellets at −70°C until lysed for analysis. Plasma specimens were aliquoted and then stored at −70°C until diluted for enzyme immunoassay. Saliva specimens were collected in cups after the patient chewed paraffin wax and were stored at 4°C for less than 24 hours before being aliquoted and then stored at −70°C until being lysed for PCR analysis. BAL and BM specimens were collected as soon as they were available and were processed similarly to blood specimens. Skin biopsies were stored at −70°C until lysed for analysis.

Cells and Viruses

The human T-cell line JJhan and phytohaemagglutinin (PHA)-stimulated cord blood lymphocytes (CBL) were used for the routine propagation of laboratory strains HHV-6A(U1102) and HHV-6B(Z29), respectively. JJhan cells and HHV-6A(U1102) were a gift from J. Black (Centers for Disease Control and Prevention, Atlanta, GA). Cord blood used for routine propagation of laboratory strains and for virus isolation studies was obtained from healthy placentas after delivery at Magee Women’s Hospital (Pittsburgh, PA).

Virus Isolation

Virus isolation was attempted only from mononuclear cells obtained from marrow or blood. Procedures for the isolation and identification of HHV-6 were as described elsewhere. Cultures were monitored weekly for growth of HHV-6 by observing the cytopathic effect. The medium was changed and fresh PHA-stimulated cord blood mononuclear cells were added weekly for up to 3 weeks. After 4 weeks, the cultures were frozen in liquid nitrogen. Virus isolation

<table>
<thead>
<tr>
<th>Table 1. Patient Characteristics</th>
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<td><strong>Patient No.</strong></td>
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<td>26</td>
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</tbody>
</table>

Abbreviations: Auto, autologous transplantation; Allo, allogeneic transplantation; AML, acute myelogeneous leukemia; CML, chronic myelo- 
geneous leukemia; NHL, non-Hodgkin’s lymphoma; ALL, acute lymphocytic leukemia; OC, ovarian carcinoma; BC, breast carcinoma; TBI, total body irradiation; VP16, etoposide; Cy, cyclophosphamide; Bu, busulfan; T, thiopeta; Cb, carboplatin.
was confirmed when cultured material from the recipient was posi-
tive by PCR for HHV-6 DNA or HHV-6-specific proteins by immu-
nofluorescence.

**Immunofluorescence Assay**

Cells from the cultures at 2 and 4 weeks were monitored for the
presence of HHV-6 by staining with p41 (9A5D12) MoAb (B0145;
Universal Biotechnology, Rockville, MD), which detects an HHV-
6 early protein using an indirect immunofluorescence method. When
cells that had been reacted with HHV-6-specific MoAb exhib-
ted dense nuclear staining and the normal mouse serum controls
showed no staining, the culture was considered positive for presence
of HHV-6 antigen. Mock-injected and HHV-6B(Z29)-infected CBL
were tested with each batch of slides.

**PCR Amplification**

Approximately 10⁶ mononuclear cells obtained from blood, BM,
or BAL were used for hot-start PCR amplification from primary
(direct PCR) and cultured material (weeks 2 and 4 of culture; cul-
tured PCR). Cells were digested in 100 μL lysis buffer (10 mmol/
L Tris, pH 8.5, 1% 10-lauryl ether with 200 μg/mL proteinase K)
at 65°C for 2 hours, followed by incubation at 98°C for 10
minutes to destroy the proteinase. Twenty-nicroliter (2 × 10⁶ cells equiva-
 lent) and 2-μL (2 × 10⁵ cells equivalent) aliquots of the cell lysate were
used for PCR amplification of HHV-6 and human β-globin
DNA using two different sets of primers in the same reaction tube.
HHV-6 2A and 2B primers were specific for HHV-6 DNA and
KM38 and PC03 primers were specific for β-globin (internal con-
trol). Reactions consisted of 50 mmol/L KCl; 10 mmol/L Tris, pH
8.5; 1.5 mmol/L MgCl₂; 0.01% gelatin (patients no. 1 through 7
only; gelatin was eliminated in the remaining patients because it
was shown to interfere with PCR reactions); 200 μmol/L each of
dATP, dTTP, dCTP, and dGTP (Pharmacia, Piscataway, NJ); 2.5 U
of Taq DNA polymerase (AmpliTag; Perkin Elmer, Norwalk, CT);
and 1.0 μmol/L of each primer. The reaction mix was incubated at
80°C for 10 minutes before the cell lysate was added to prevent
nonspecific amplification. Thermocycling conditions consisted of
incubation at 90°C for 1 minute, followed by 35 amplification cycles,
each consisting of denaturation at 94°C for 1 minute, annealing
at 55°C for 2 minutes, and extension at 72°C for 1 minute. This
was then followed by an extension step at 72°C for 7 minutes.
Specific amplification products were 187 bp and 167 bp for HHV-6 and
β-globin, respectively. Amplified products were electrophoresed in
composite agarose gels (3% Nusieve GTG agarose and 1% SeaKem
agarose; FMC, Rockland, MD) for 3 hours at 1 V/cm (electrode
to electrode) in TBE at room temperature, washed with phosphate-buffered saline
(PBS-bovine serum albumin (PBS-BSA) to block nonreacted sites.
Plasma specimens were diluted 1:100 in serum diluent (PBS, pH
7.4, 1% BSA, 0.05% Tween-20, and 2% [vol/vol] of a normal cell
culture antigen prepared from the HEL strain of human lung fibro-
blasts), added to two infected and two uninfected wells, and incu-
bated for 4 hours at 37°C in 5% CO₂.
IgG (γ) (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD). After washing, bound antigen-antibody complex was detected with alkaline phosphatase substrate. Optical density (OD) was read at 405 nm with a microplate reader. The net absorbance value for each serum sample was determined by subtracting the OD of the control wells from the OD of the viral antigen wells. The final EIA value for each sample was determined by a calibration procedure using four standardized control serum samples on each plate and standard linear regression analysis. The EIA value is proportional to the amount of antibody in the test plasma. A cut-off value of 0.080 was used to determine positivity.31 Plasma specimens were collected at two time points before transplantation to perform serologic analyses (baseline titers). A change in titer was considered significant if there was a 1.6-fold increase or decrease in the titer as measured by EIA OD value, from baseline titer determined at day −1 (or day 0, before the transplant).

Criteria Established to Determine Association of Active HHV-6 Infection With Disease Events and Activation of Other Herpesviruses

The following terms were used to define the relationship between the virus and the host. All patients were seropositive to HHV-6 (baseline titers). Isolation of HHV-6 from blood, detection of HHV-6 DNA in the saliva, or a significant increase in HHV-6-specific antibody titers were considered as evidence of "active" HHV-6 infection. HHV-6 disease was defined as the presence of signs or symptoms attributed to active HHV-6 infection.

The clinical events examined for their relationship to active HHV-6 infection were fever, skin rash, GVHD, pneumonia, ARDS, engraftment, marrow suppression, CNS involvement, sinusitis, oral mucositis, gastroenteritis, bacterial infection, and reactivation of other herpesvirus infections: herpes simplex virus (HSV), varicella zoster virus (VZV), and HCMV.

Associations were established between clinical events and active HHV-6 infection occurring at any time point during the study period using Fisher’s exact test (two-tailed), and the Pearson χ² test for contingency tables, linear regression analysis, Kaplan-Meier survival analysis, and the Log Rank test, were performed using the STATA program (STATA Corp, College Station, TX).

RESULTS

Patient Population

Twenty-six patients were observed for a median of 118 days (range, 16 to 328 days). The median follow-up periods in allogeneic and autologous transplantation patients were 90 days (range, 19 to 327 days) and 260 days (range, 58 to 328 days), respectively. The mortality rate was higher in the allogeneic as compared with the autologous transplant patients (P = .03; Fig 1). The median survival time was 102 days.
Table 3. A Summary of Active HHV-6 Infections in the Study Population

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Type of Transplantation</th>
<th>HHV-6 Isolation From Blood</th>
<th>HHV-6 DNA in Saliva</th>
<th>Posttransplantation HHV-6 Serologic Increase</th>
<th>Active Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Allo, MRD</td>
<td>+ (90)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Allo, MRD</td>
<td>+ (48, 153)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Allo, MRD</td>
<td>–</td>
<td>+ (0, 7, 122, 171)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Allo, MRD</td>
<td>+ (132)</td>
<td>+ (6, 14, 20, 27, 62, 83, 106, 132, 140)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Allo, MRD</td>
<td>–</td>
<td>+ (64)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>Allo, MRD</td>
<td>–</td>
<td>+ (–7, –1)</td>
<td>+ (6)</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>Allo, MRD</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>25</td>
<td>Allo, MRD</td>
<td>–</td>
<td>+ (–1, 13, 20, 24, 62, 90)</td>
<td>+ (13)</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Allo, MUD</td>
<td>–</td>
<td>+ (–6)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Allo, MUD</td>
<td>+ (24, 27)</td>
<td>–</td>
<td>+ (27)</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Allo, MUD</td>
<td>+ (50)</td>
<td>+ (7, 14, 21, 29, 50)</td>
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<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Allo, MUD</td>
<td>–</td>
<td>+ (0)</td>
<td>+ (20)</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>Allo, MUD</td>
<td>+ (68)</td>
<td>+ (–8, –2, 12, 20, 27, 68)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>Allo, MUD</td>
<td>–</td>
<td>+ (–6, 21, 28, 54)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>23</td>
<td>Allo, MUD</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Auto, BM</td>
<td>+ (21)</td>
<td>+ (–7)</td>
<td>+ (21)</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Auto, BM</td>
<td>+ (15, 324)</td>
<td>+ (–7, –2, 15, 26)</td>
<td>–</td>
<td>+</td>
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<tr>
<td>16</td>
<td>Auto, BM</td>
<td>–</td>
<td>+ (50, 81)</td>
<td>–</td>
<td>–</td>
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<tr>
<td>19</td>
<td>Auto, BM</td>
<td>–</td>
<td>+ (–7, 13, 41)</td>
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<tr>
<td>24</td>
<td>Auto, BM</td>
<td>+ (28)</td>
<td>+ (28, 92)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Auto, PBSC</td>
<td>+ (198)</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>12</td>
<td>Auto, PBSC</td>
<td>+ (13)</td>
<td>–</td>
<td>+ (38)</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Auto, PBSC</td>
<td>–</td>
<td>–</td>
<td>+ (30)</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>Auto, PBSC</td>
<td>+ (20, 65)</td>
<td>+ (13)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>Auto, PBSC</td>
<td>–</td>
<td>0</td>
<td>–</td>
<td>+</td>
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</table>

All patients were seropositive to HHV-6 before transplantation. Numbers in parentheses are the days from transplantation.

Abbreviations: Auto, autologous transplantation; Allo, allogeneic transplantation.

days in allogeneic and greater than 182 days in autologous transplantation patients.

Direct Detection by PCR

Detection of HHV-6 DNA was attempted by direct PCR as well as by PCR on material cultured from transplant recipients (patients) and donors. Mononuclear cells were isolated from heparinized blood, marrow obtained from both donors and patients, and BAL from patients. One million cells of primary material were saved for direct PCR when possible. Primary material (mononuclear cells) was always negative when tested for HHV-6 DNA. In some patients, particularly the allogeneic transplantation patients who took longer to engraft, as few as 1 x 10^6 cells were available. If more than 1 x 10^6 cells were obtained, 1 x 10^6 were saved for PCR and the remainder were cultured.

Virus Isolation

HHV-6 was identified by PCR in cultured PBMCs from 12 of 26 patients (Table 3). In 4 of these 12 patients, virus was isolated on two occasions (2 autologous and 2 allogeneic). In total, 16 isolates of HHV-6 were obtained. When cultures from patients no. 1 through 7 were analyzed, all the cultures positive at week 2 were also positive at week 4 of the culture. PCR was therefore performed only on primary material and material cultured for 4 weeks in patients no. 8 through 26. For all of the patients from whom virus isolation was achieved, direct PCR was negative and only the cultured material was positive by PCR. A representative PCR analysis performed on patient no. 12 is shown in Fig 2. Of the 5 cultures established from patient no. 12 at days 6, 13, 20, 38, and 101 after transplantation, only the culture from day 13 was positive for HHV-6 DNA.

There were equal numbers of patients (6) with virus isolation in the autologous and allogeneic transplantation groups. Differences in the frequencies among the four subgroups of transplantation patients studied here were not statistically significant. In addition, we found no correlation between the frequency of HHV-6 isolation and the number of cells tested.

The numbers of cells cultured were sorted into ascending range and divided into equal numbers of cultures in the lower cell range (2.5 x 10^4 to 2.2 x 10^5, n = 111) and higher cell range (2.3 x 10^5 to 3.7 x 10^6, n = 110). Cultures in the higher cell range were more frequently PCR positive (5/111 vs 11/110), but the difference was not statistically significant (P = .128, Fisher’s exact test).

In addition to performing PCR to identify HHV-6, IFA was also performed on the cultured material at weeks 2 and 4 of culture. HHV-6 was identified by IFA in 8 of 12 PCR-
positive cultured PBMCs. Cultures from patients no. 2, 6, 9, and 22 on days 198, 153, 324, and 65, respectively, were PCR positive but IFA negative. Thus, PCR was more sensitive (16/221 cultures positive) than IFA (12/221 cultures positive) in detecting the presence of HHV-6 in cultured material ($P < .05$, McNemar’s test).

Salivary Shedding of HHV-6 DNA

Eleven of the 26 patients (42%) showed evidence of salivary shedding of HHV-6 before transplantation (Table 3). Posttransplantation analysis was limited to the 19 patients who were studied longitudinally. Twelve of 19 patients (63%) were positive for salivary shedding after transplantation. Posttransplantation, there was a statistically significant difference in the frequency of HHV-6 shedding between allogeneic (30/56 specimens [53%]) and autologous (9/31 specimens [29%]) patients ($P = .042$). Pretransplantation, no such difference was observed, with the frequencies being 7 of 18 specimens (38%) in allogeneic and 4 of 13 specimens (30%) in autologous transplantation patients ($P > .05$).

A statistically significant difference was observed between the number of saliva specimens positive in the autologous transplantation patients who received BM (11/23 [47%]) when compared with those who received PBSCs (2/21 [9%]; $P = .008$). No such difference was observed within the two subgroups of allogeneic transplantation patients, ie, those who received BM from matched related donors (22/41 [53%]) and those who received BM from matched unrelated donors (15/33 [45%]).

A significant negative association was observed between the fraction of saliva specimens with HHV-6 DNA and the mean HHV-6 antibody levels in the 19 patients in whom salivary shedding was observed longitudinally ($P = .005$, linear regression analysis).

HHV-6 Serology

All of the recipients and donors were seropositive for HHV-6 at the time of transplantation. A significant increase in HHV-6 antibody titer was seen in 7 of 26 (26%) recipients (Table 3). Four of these were allogeneic and the remaining three were autologous transplantation patients. In subjects in whom a significant change in antibody levels was seen, the median time to the increase in titer was 21 days (range, 6 to 38 days). Significant decreases in titer were seen in 5
of 26 patients at a median time of 31 days (range, 14 to 140 days). No significant difference in the proportion of patients with increases in HHV-6 antibody titers were observed between allogeneic and autologous transplantation patients. Moreover, no statistically significant association was observed between virus isolation and increases in HHV-6 antibodies \((P > .05)\).

**Frequency of Active HHV-6 Infection**

BMT increases the frequency of HHV-6 isolation as shown by a significant proportion of cultures being positive after transplantation when compared with the absence of positive cultures before transplantation \((P = .007; \text{Table 4})\). As described above, differences in the proportion of saliva specimens positive during pretransplantation and posttransplantation periods were not statistically significant. The posttransplantation period was further divided into early posttransplantation \((\text{days 0 through 16)}\) and late posttransplantation \((>16 \text{ days)}\) based on the median time to neutrophil engraftment \((\text{day 16)}\). No significant difference was observed in the proportion of cultures and saliva positive between the early and late posttransplantation period \((\text{Table 4})\).

The effects of therapy with the antivirals acyclovir and ganciclovir on HHV-6 isolation from cultures and its detection in saliva were studied by determining the proportion of cultures positive during acyclovir treatment and no antiviral treatment \((\text{Table 4})\). The number of cultures positive during acyclovir treatment was significantly lower than that during periods with no acyclovir treatment. However, no significant difference was observed during ganciclovir treatment.

**Detection of HHV-6 DNA in Marrow, BAL, Urine, and Biopsy Specimens**

BM specimens from donors of allogeneic transplants as well as from autologous transplantation patients before transplantation were negative for virus isolation. BAL specimens were obtained from only 2 recipients and were also negative. No HHV-6 DNA was detected in urine specimens from the first seven patients before transplantation. Thirty skin biopsies tested from a total of 12 patients were negative for HHV-6 DNA, except for 1 time point in patient no. 1. In that patient, the skin rash was considered to be a manifestation of GVHD. It is possible that, because HHV-6 DNA was present in the biopsy material, the skin rash was related to HHV-6 infection. In addition, HHV-6 DNA was detected by PCR in lung tissue obtained from patient no. 19 at autopsy. No other identifiable cause was found for the pneumonia and subsequent development of ARDS and death. Therefore, HHV-6 may have been involved in the disease in this patient.

**Variant Analysis of HHV-6 Isolates**

**RFLP of PCR amplimers.** In the present study, all the cultures that were positive with primer set HHV-6 2A and 2B were also positive with the Aubin primers, except for the culture from patient no. 22 at day 65. In addition, restriction profiles of the PCR amplifiers from the HindIII and HindI digestions were characteristic of variant B.7 Thus, by both HindIII and HindI digestion, all of the isolates of HHV-6 were the B variant.

**MoAb analysis.** As discussed earlier in the section on detection of HHV-6 by immunofluorescence, four PCR-positive cultures were negative by IFA. The remaining cultures reacted with the panel of MoAbs in a manner consistent with variant B specificity.

**Heterogeneity Among Viral Isolates Studied by RFLP Analysis of Viral DNA**

To show that the HHV-6 isolates were different from the laboratory strain HHV-6B(Z29) and were not cross-contaminants from isolates obtained from other patients, RFLP studies were performed on materials from the patients for whom sufficient infected cells were available \((\text{Fig 3)}\). As can be seen in Fig 3, although there are numerous differences between HHV-6B(Z29) and the BMT isolates, as well as among the BMT isolates, they all exhibited variant B patterns.11 The two isolates from the same patient \((\text{patient no. 7, days 24 and 27)}\) were identical to each other but differed from the other isolates, as shown by restriction profiles with enzymes BamHI and Pst I.

**Associations With Disease**

All patients were seropositive to HHV-6 before BMT and thus all patients were considered to be infected with HHV-6. During prospective clinical observation, no episodes of identifiable HHV-6 disease were directly attributed to active HHV-6 infection. After the observation period, clinical data collected from all of the patients were assessed for associations with episodes of active HHV-6 infection.

Table 5 shows the frequency of the various clinical events as well as documented infections observed in the patients under study. Data collected on bacterial infections were useful in interpreting either febrile events or pneumonia. Diarrhea and oral mucositis occurring early in the posttransplantation period...
Fig 3. Southern blot analysis of HHV-6 isolates. Restriction fragment polymorphism studies were performed on HHV-6 isolates using restriction endonucleases *NciI*, *PstI*, *SalI*, and *BamHl* on whole cell DNA preparations. HHV-6B (Z29) was included as a positive control. The blots were probed with HHV-6 nucleocapsid DNA. The restriction profile of all of the isolates differed from each other and the laboratory strain HHV-6B (Z29).

Simple Associations of Active HHV-6 Infection to Disease Events

A high proportion of patients had one or more manifestations of active HHV-6 infection. Tests were also performed separately to detect associations with virus isolation from blood, the presence of viral DNA in saliva, and significant serologic increases to HHV-6. Associations with fever, skin rash, GVHD, pneumonia, ARDS, and CNS disease were not significant. Sinusitis was significantly associated with HHV-6 isolation in autologous transplant patients (*P* = .002). Active HCMV infection was significantly associated with HHV-6 isolation in allogeneic transplantation patients.

Temporal Association of HHV-6 Infection With Disease Events

A summary of the temporal association of active HHV-6 infection to clinical events with a scoring for the possible causal relationship is shown in Table 6. HHV-6 infection was a “probable” cause of fever in 4 patients, skin rash in 3 patients, fever associated with skin rash in 3 patients, sinusitis in 2 patients, pneumonia with ARDS in 1 patient, and platelet and RBC suppression in 1 patient. In all, 9 of the 26 patients had some clinical event probably due to HHV-6 infection, as defined by events occurring within 2 weeks of documented infection in the absence of other demonstrable causes. When the data of Table 6 (excluding marrow suppression) were tested for independence of columns (association scores) and rows (clinical events), no association was observed (*P* = .773, Pearson *χ*² test). The

<table>
<thead>
<tr>
<th>Patient No.</th>
<th><em>NciI</em></th>
<th><em>PstI</em></th>
<th><em>SalI</em></th>
<th><em>BamHl</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-6B (Z29)</td>
<td>1/7/7/9/10/22</td>
<td>6/7/7/9/10/22</td>
<td>7/7/9/10/22</td>
<td>7/7/9/10/22</td>
</tr>
</tbody>
</table>

in the absence of cultured herpes simplex virus were considered to be gastrointestinal toxicity due to chemotherapy.

Most of the patients experienced febrile episodes after transplantation (73%; Table 5). A high frequency of skin rash was observed in allogeneic transplantation patients (80%) compared with autologous patients (36%). Pneumonia and ARDS episodes were generally limited to allogeneic patients (60%), except for one autologous transplantation patient. Graft failures were limited to allogeneic patients receiving marrow from matched unrelated donors. Sinusitis episodes were observed at a higher frequency in autologous (55%) than in allogeneic transplantation patients (21%). CNS involvement manifested as encephalopathy or neuropathy was again observed at a higher frequency in allogeneic (21%) than in autologous transplantation patients (9%).
marrow suppression data at the bottom of the Table 6 was analyzed separately, because the defining criteria were different from those for the clinical events in the upper part of the table; no association was observed (P = .860).

HHV-6 DNA was detected in the skin biopsy specimen of 1 patient with GVHD histologically diagnosed in the same specimen. Thus, HHV-6 was considered to be only a “possible” cause of skin rash in this case. In another patient, HHV-6 DNA was detected by PCR in lung tissue obtained at the time of autopsy. This patient died of idiopathic pneumonia and HHV-6 was therefore considered as the “probable” cause of the pneumonia.

Effect of HHV-6 Infection on Marrow Engraftment and Suppression

Figure 4 shows the time course of engraftment for patients with and without HHV-6 isolation from the blood. There was no statistically significant difference in the rate of engraftment or frequency of patients with engraftment in any of the 3 lymphoid lineages in patients with virus isolation when compared with those without virus isolation (Fig 4, P > .05 by log rank test). The median times to neutrophil, platelet, and RBC engraftment were 16, 30, and 51 days, respectively, in patients with no virus isolation. The median times to neutrophil, platelet, and RBC engraftment in patients with virus isolation was 17, 58, and greater than 26 days, respectively.

No statistically significant association was observed between active HHV-6 infection and neutrophil, platelet, or RBC suppression. Of the 22 patients that were evaluated for neutrophil suppression, 9 showed suppression. Active HHV-6 infection manifested by virus isolation was identified by the propinquity analysis as a “possible” and “improbable” cause of neutrophil suppression in 3 and 6 patients, respectively. Of the 15 patients evaluated for platelet suppression, 9 showed suppression. Active HHV-6 infection manifested by virus isolation was identified as the “probable,” “possible,” and “improbable” cause of platelet suppression in 1, 2, and 6 patients, respectively. Of the 13 patients evaluated for RBC suppression, 8 showed suppression. Active HHV-6 infection manifested by virus isolation was identified as a “probable,” “possible,” and “improbable” cause of RBC suppression in 1, 2, and 5 patients, respectively (Table 6).

Activation of Other Herpesviruses

HCMV. Five of the 26 recipients were seronegative to HCMV and 21 were seropositive to HCMV before trans-
plantation. None of the five seronegative patients showed any evidence of HCMV infection after transplantation. Four of the five (80%) showed active HHV-6 infection after transplantation (Table 7). There was no difference in the frequency of active HHV-6 infection among HCMV-seronegative patients (80%) and HCMV-seropositive patients with or without evidence of posttransplantation activity (88% and 91%, respectively). HHV-6 was isolated from the peripheral blood at a higher frequency in patients with active HCMV infection (8/12 [66%]) than those with no evidence of active HCMV infection (3/14 [21%]), the difference being statistically significant \( P = .04 \) in allogeneic transplantation patients.

**HSV and VZV.** In 6 of 26 (23%) and 2 of 26 (7%) recipients, active HSV and VZV infection were documented, respectively (Table 7). In 5 of 6 cases, active HSV infection followed active HHV-6 infection; in 1 of 6 recipients, it preceded active HHV-6 infection. One of the two cases of active VZV infection followed HHV-6 infection, whereas the other preceded HHV-6 infection. No statistically significant association was observed between active HHV-6 infection and activation of HSV or VZV.

**DISCUSSION**

**HHV-6 in BMT Recipients**

The present study involved 26 adult BMT patients (11 autologous and 15 allogeneic) observed prospectively for up to 1 year after transplantation. Previous studies examining the frequency of HHV-6 infections were nearly exclusively limited to allogeneic transplantation patients because they are more immunosuppressed than the autologous transplantation patients and are considered to be at a greater risk of primary as well as reactivated herpesvirus infections.\(^ {16,18} \) In addition, in the studies by Yoshikawa et al\(^ {18} \) and Drobsky et al.\(^ {16} \), 25 pediatric BMT patients (24 allogeneic and 1 autologous) and 16 adult BMT patients (all allogeneic) were observed for 60 and 100 days, respectively. Drobsky et al\(^ {16} \) routinely attempted HHV-6 isolation only after the first 3 weeks after transplantation; thereafter, HHV-6 isolation was attempted only during febrile episodes with no identifiable cause. In our study, we examined all the patients routinely as per the experimental design unless the patient died or was lost to follow-up; both autologous and allogeneic transplantation patients were studied. Thus, this study differs from previous studies with respect to study design and duration of follow-up, as well as the composition of the transplantation population.

HHV-6 was isolated from the cultured blood mononuclear cells of 12 of 26 (46%) patients during the study period, which is comparable to the frequency reported by others, ie, 10 of 25 patients (40%)\(^ {13} \) and 6 of 16 patients (37%).\(^ {15} \) In the remaining two studies, HHV-6 was isolated from 3 of 3 patients (100%)\(^ {15} \) and 2 of 2 patients (100%).\(^ {14} \)

In the present study, all patients and donors were HHV-6 seropositive but culture negative before transplant. Three of the 12 patients (25%) from whom virus was isolated also showed significant increases in HHV-6-specific antibody titers after transplantation, as measured by enzyme immunoassay. This is lower, but not statistically different from that observed by Yoshikawa et al\(^ {18} \) and Asano et al.\(^ {13} \), in which 5 of 10 patients (50%) and 2 of 3 patients (66%) with virus isolation, respectively, showed fourfold increases in the neutralizing antibody titers. In addition, in the present study, 4 of 14 patients (29%) from whom no virus was isolated also showed significant increases to HHV-6 after transplantation. This is a higher proportion than that observed by Yoshikawa et al.\(^ {18} \), in which 2 of 15 patients (13%) with no virus isolation showed 16-fold increases in the neutralizing antibody titers. In total, 7 of 26 of the patients (27%) in the present study showed significant increases in HHV-6-specific antibody titers, which is similar to the result observed by Yoshikawa et al.\(^ {18} \). Because allogeneic BMT patients are compromised in their humoral responses, the frequency of infection may be underestimated by analysis of serology. Therefore, serology

![Fig 4. Establishment of time to engraftment in 3 different lineages (ANC, platelets, and RBC) in the presence or absence of HHV-6 isolation using Kaplan-Meier survival analysis. (–) Patients with engraftment (ANC, platelet, and RBC) in absence of virus. (…) Patients with engraftment in presence of virus isolation. Significance was determined by log rank test, and in each of the 3 cases \( P > .05 \).](http://www.bloodjournal.org/content/53/18/5350)
HHV-6 IN BONE MARROW TRANSPLANTATION

Table 7. Activation of Human Herpesviruses in the Study Population

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Type of Transplantation</th>
<th>Pre-Tx HCMV Serology</th>
<th>Active HCMV Infection</th>
<th>Active HSV Infection</th>
<th>Active VZV Infection</th>
<th>Active HHV-6 Infection</th>
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<tbody>
<tr>
<td>1</td>
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<td>+</td>
<td>V</td>
<td>-</td>
<td>-</td>
<td>V</td>
</tr>
<tr>
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<td>S, S</td>
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<tr>
<td>18</td>
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<td>S, S</td>
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<tr>
<td>19</td>
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<td>S</td>
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</tr>
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<tr>
<td>21</td>
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<td>+</td>
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<tr>
<td>22</td>
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<td>+</td>
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<td>-</td>
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<td>+</td>
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<tr>
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<tr>
<td>26</td>
<td>Auto, PBSC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>S</td>
</tr>
</tbody>
</table>

Abbreviations: Auto, autologous transplantation; Allo, allogeneic transplantation; V, virus isolation; A, significant antibody increases after transplantation; S, salivary shedding of HHV-6 DNA.

cannot be used as a sole method in documenting active HHV-6 infection. It is also possible to show false-positive serology in patients receiving large volumes of IVIG transfusion. We did not observe any effect of IVIG transfusion on serologic analysis (data not shown).

Direct PCR

We were unable to detect HHV-6 DNA by direct PCR of mononuclear cells with primer set HHV-62A/2B. However, previous studies have shown the presence of HHV-6-specific DNA in the PBMCs from healthy adults. The differences between the above two studies and this study could be the number of cells tested and the primer sets used for PCR analysis. Jarrett et al. showed that DNA equivalent to $5 \times 10^5$ to $10^6$ cells was required to detect HHV-6-specific DNA in the PBMCs. Cone et al. tested DNA obtained from 7.5 mL of blood (approximately $7 \times 10^6$ cells). In our study, we tested a median of $1.93 \times 10^5$ by direct PCR from the 16 blood specimens that were PCR positive on culture. Furthermore, we used a different primer set for PCR analysis. It is possible that our primer set is not as sensitive in detecting HHV-6 DNA as those used by others. Support for the latter hypothesis comes from the observation that primary materials from patients no. 6 and 17 at days 48 and 68, respectively, were negative when tested by PCR with the primer set H62A/2B but were positive when tested with primer set described by Aubin et al on ethidium bromide staining. On the other hand, the culture from patient no. 22 on day 65 was positive with primer set H62A/2B but negative with the primer set described by Aubin et al. Direct PCR of blood cells from seropositive subjects would presumably be positive in all cases if sufficient numbers of cells were tested and, unless quantitative, probably provides little information. Virus isolation, which we assume detects productively infected cells or, perhaps, free virus, may provide a significant measure of active infection, albeit at the expense of considerable effort.

Viral isolates obtained from patient no. 7 at two different time points (days 24 and 27) after transplantation were identical to each other. No virus was isolated during the pretransplantation period from the recipients or from the donors in this study, which prevented transmission studies.

Effect of Antibody on Salivary Shedding

In addition to virus isolations and significant increases in HHV-6 titers, we tested for HHV-6 DNA in saliva. This has not been previously studied in transplantation patients. The HHV-6 DNA detected in the saliva may be cell associated and may not represent active HHV-6 infection. Jarrett et al. and Cone et al. have reported that 18 of 20 (90%) and 16 of 17 (85%) healthy adults, respectively, had HHV-6-specific DNA in their saliva specimens and that 10 of 10 and 14 of 17 individuals, respectively, remained positive for HHV-6 DNA in their saliva specimens upon subsequent testing.
Infectious salivary HHV-6 has been detected only infrequently.35,36 Jarrett et al34 observed no correlation between antibody titer and the ability to isolate HHV-6 or detect HHV-6 DNA. In 15 of 19 patients (78%) from this study, there was salivary shedding at least once and usually on multiple occasions, and it was shown to have a significant negative association with the mean antibody titer. Although other investigators have not seen a correlation between HHV-6 shedding and HHV-6-specific antibody titer,35 it is important to note that none of the previous studies observed the subjects as intensively as in this study. Also, it is important to study healthy adults longitudinally to show if the correlation with the antibody titer is seen in this group as well.

Association of Active HHV-6 Infection With Disease

Active HHV-6 infection has been previously associated with several clinical events in the setting of solid organ transplantation as well as BMT, including fever, skin rash, pneumonia, and BM suppression.13,15,18,23 We examined associations between active HHV-6 infection between these and other clinical events. Of the associations tested, HHV-6 isolation was found to be significantly associated only with sinusitis (P = .002) and active HCMV infection (P = .04) in autologous and allogeneic transplantation patients, respectively.

The association of sinusitis with active HHV-6 infection is surprising. However, the virus has been associated with sinusitis as well as otitis media.23 Three allogeneic transplantation patients also had episodes of sinusitis, but the association with active HHV-6 infection was not statistically significant. Possibly, a higher frequency of sinusitis may have been missed as these patients experience a greater number of concurrent complications. In addition, the lack of other identifiable causes of sinusitis might be due to difficulty in obtaining appropriate cultures from the sinuses in neutropenic and thrombocytopenic patients. Nevertheless, the temporal association of HHV-6 infection and the onset of sinusitis is intriguing and bears further investigation. In allogeneic transplantation patients, active HCMV infection was also shown to be significantly associated with HHV-6 isolation, suggesting coactivation of these two viruses under immunosuppressed conditions.

Based on temporal association of active HHV-6 infection with clinical events, HHV-6 infection was identified as the probable cause of fever, skin rash, sinusitis, pneumonia, ARDS, platelet suppression, and RBC suppression in 9 of 26 patients. We resorted to the term probable because an unequivocal association could not be made.

We found no simple association of active HHV-6 infection with GVHD. However, the high frequency of active HHV-6 infection in our patient group would hide any direct association with GVHD. Wilborn et al17 detected HHV-6 DNA at a higher frequency (urine, buffy coat, or oral lavage fluid) in BMT patients with moderate or severe GVHD than in those with mild or no GVHD. This could be explained by increased viral activity during a more immunosuppressed state. We found no statistically significant association between GVHD and active HHV-6 infection. Nonetheless, from one patient with histologically diagnosed GVHD, HHV-6 DNA was detected in a skin biopsy, suggesting a possible etiologic association between HHV-6 and skin rash or GVHD. Previously, HHV-6 DNA has been detected in the skin biopsy of BMT patients with exanthematous rash.37,38 In both of the above studies, patients had exanthematous rash at the time of skin biopsy. These studies and our study suggest that a virologic analysis should be performed on skin biopsy in addition to the pathologic analysis performed for GVHD. Although a high frequency of pneumonia episodes was observed in allogeneic (12 of 15) compared with autologous (1 of 11) transplantation patients, no simple association was observed between active HHV-6 infection and development of pneumonitis. However, there was one patient in whom HHV-6 DNA was found in the lung tissue obtained at autopsy and who died of idiopathic pneumonia.

It has been suggested that HHV-6 might be responsible for idiopathic pneumonia after BMT14,15 and delays in engraftment and marrow suppression.16 However, in our study population, there was no statistically significant association between active HHV-6 infection and either delayed engraftment or marrow suppression in any of the 3 lineages studied (neutrophil, platelet, and RBC). However, in two patients, HHV-6 was considered as a probable cause of platelet and RBC suppression.

The Role of Immunosuppression

The frequency of HHV-6 shedding in saliva may also be dependent on the degree of immunosuppression. Thus, allogeneic transplantation patients, who are more immuno-suppressed, showed significantly higher levels of salivary shedding compared with autologous transplantation patients (P = .035). The correlation noted above between the frequency of salivary shedding and antibody levels was due to the contribution of autologous transplantation patients (P < .05, linear regression analysis) but not allogeneic transplantation patients (P > .05). The lack of correlation observed in the case of allogeneic transplantation patients might be due to passive antibodies received by those patients in the form of IVIG transfusions for prophylaxis of HCMV disease. No IVIG transfusions were administered to autologous transplantation patients after transplantation unless the patient developed HCMV disease.

There was no statistically significant difference in salivary shedding between allogeneic transplantation patients receiving marrow from living related donors and matched unrelated donors, but a significant difference was observed between autologous transplantation patients who received marrow versus those who received PBSCs. The difference in the latter two groups is that, in patients receiving marrow, immune reconstitution takes place from the progenitor cells alone, whereas in the patients receiving PBSCs, immune reconstitution is due to stem cells with the addition of mature leukocytes of a variety of lineages, which accelerates engraftment.

The Role of Antivirals

In our study, the number of PCR-positive cultures was significantly lower during periods of acyclovir treatment
when compared with periods with no acyclovir treatment. This suggests that acyclovir has some antiviral activity against HHV-6 in BMT recipients. These results are counter to expectation, in as much as ganciclovir has a marked effect on HHV-6 in vitro, whereas acyclovir has much less effect. Similar observations have been made with respect to effect of acyclovir on HCMV infection and disease. Acyclovir has no significant effect on HCMV in cell cultures and patients. However, a study by Meyers et al in BMT patients showed that patients receiving acyclovir treatment were shown to have significantly decreased levels of HCMV disease when compared with patients with no acyclovir treatment. This suggests that acyclovir may have an antiviral effect on both HHV-6 and HCMV, the mechanism of which is not yet understood.

**HHV-6 Variants**

Similar to the observations of Drobyski et al, all the HHV-6 isolates from this study were variant B. HHV-6B consists of two subgroups of viruses on the basis of restriction pattern of PCR amplimers. In this study, we identified representatives of both classes.

In summary, active HHV-6 infections occurred frequently after transplantation. Virus isolation with PCR confirmation is more sensitive than monitoring cultures by IFA. There was no statistically significant difference between the overall frequency of active HHV-6 infection, manifested as virus isolation, increases in HHV-6-specific antibody titer, and salivary shedding of HHV-6 DNA, between allogeneic and autologous transplantation patients. Because of the relatively high frequency of active HHV-6 infection in this group of patients, simple temporal correlations with clinical events are not sufficient to establish causality until the spectrum of HHV-6 disease has been more clearly defined. Nevertheless, active HHV-6 infection was implicated as a potential cause of disease in a substantial proportion of our patients. A definitive correlation would require a much larger sample size than our study allowed. Despite these limitations, our findings suggest that HHV-6 infection and disease should be important considerations in assessing patients after BMT and that further studies are warranted for the pathogenic role of HHV-6 in BMT patients.

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Human herpesvirus 6: infection and disease following autologous and allogeneic bone marrow transplantation

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