Early Occurrence of Human Cytomegalovirus Infection After Bone Marrow Transplantation as Demonstrated by the Polymerase Chain Reaction Technique

By Hermann Einsele, Michael Steidle, Angelika Vallbracht, Johannes G. Saal, Gerhard Ehniger, and Claudia A. Müller

Twenty-eight patients undergoing bone marrow transplantation (BMT) were followed-up at weekly intervals from day −10 to discharge from hospital after BMT for human cytomegalovirus (HCMV) infection using polymerase chain reaction (PCR), slot-blot hybridization, and conventional virus culture. High specificity of the PCR assay applied could be shown by failure to amplify DNA extracted from a wide range of other viruses frequently infecting marrow transplant recipients. The PCR technique allowed us to diagnose viremia and viruria in 20 (83%) of 24 seropositive patients after BMT, whereas culture assays showed 16 (67%) of 24 of these patients to be viruric and 9 (37%) of 24 cases to be viremic. Slot-blot hybridization showed a frequency of viruria and viremia in 12 (50%) of 24 seropositive patients. By application of PCR techniques, HCMV detection could be achieved even in the very early posttransplant period. HCMV was detected in five patients even before the onset of clinical symptoms of acute graft-versus-host disease. Analysis by PCR techniques of 33 organ biopsy specimens from patients after BMT showed the presence of HCMV in 13 of 14 liver samples obtained from patients with HCMV viremia; three liver specimens from patients without viremia were negative by all the techniques applied. HCMV could also be demonstrated in postmortem lung biopsy specimens from all patients (n = 10) with interstitial pneumonia.

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ALLOGENEIC BONE MARROW transplantation (BMT) is frequently complicated by human cytomegalovirus (HCMV) infection in association with severe or even fatal graft-versus-host disease (GVHD).1 Although in animals induction and aggravation of acute GVHD by cytomegalovirus have been demonstrated,2 in clinical studies,3,4 despite serologic evidence5 no temporal relationship between HCMV infection and acute GVHD (aGVHD) could be shown. With the use of conventional virologic assays, such as virus culture, HCMV infection cannot usually be diagnosed earlier than 6 to 8 weeks after BMT.5 Serologic tests for the detection of specific IgG or IgM antibodies are also not reliable in these patients because of the applied transfusion therapies or the still-defective humoral antiviral immune responses. For an early prevention and consequent therapy of severe clinical symptoms, as well as for an evaluation of the role of the virus in the pathogenesis of graft rejection or GVHD, faster and more sensitive diagnostic techniques of HCMV infection are required in these patients.

Recent studies established higher sensitivity of hybridization compared with virus culture techniques for HCMV detection in clinical specimens.5,6 Further improvement in virus detection was achieved by the introduction of the polymerase chain reaction (PCR) allowing highly specific amplification of viral DNA from infected tissues.6 Thus, diagnosis of human immunodeficiency virus (HIV) infection could be established several months before positive reactions in serologic assays.9

In this study, PCR, in association with a radioactive HCMV-DNA detection system, was evaluated for its sensitivity and practical value in the diagnosis of HCMV infection after BMT in comparison with conventional culture tests and slot-blot and in-situ hybridization assays. The main aim of the study was to determine whether application of these techniques applied shortly after BMT might allow detection of the virus earlier than reported up to now in the posttransplant period.

MATERIALS AND METHODS

Patients and clinical specimens. Twenty-eight recipients of an allogeneic BMT were followed-up for HCMV infection by different techniques until discharge from hospital (median: 10 weeks after BMT). Five patients died during in-patient treatment after BMT. Patient characteristics are given in Table 1. Only four of the patients studied were seronegative for HCMV and received transplants from seronegative donors as well as seronegative blood products. All the patients received oral acyclovir (4 × 400 mg/day) and all the seropositive patients additionally HCMV hyperimmune globulin every third week beginning on day −10 until day +100 after BMT.

Clinical specimens analyzed are shown in Table 2. Six percutaneous and postmortem liver biopsy samples and four postmortem lung biopsy specimens obtained from the patients prospectively studied were examined for the presence of HCMV. To increase the number of samples for this analysis another 12 postmortem lung biopsy samples (six from patients with interstitial pneumonia) and 11 liver samples cryopreserved from patients after allogeneic BMT not included in the prospective analysis were studied. All the samples were cryopreserved until used.

Informed consent was obtained from all patients or their parents to use a part of the clinical material obtained for routine diagnostic evaluation in this study. Approval for the study was also obtained from the local ethic committee.

Virus strains and control cells. The laboratory HCMV strain AD169, as well as 50 different clinical isolates of HCMV, were used for amplification by PCR. Most of the wild-type HCMV isolates were obtained from organ transplant recipients, others were derived from children with acquired HCMV infection. To control for nonspecific amplification of other viral DNA fragments, low-passage clinical isolates of varicella-zoster-virus (VZV), herpes simplex virus type I and II, several strains of adenoviruses, as well as for nonspecific amplification of other viral DNA fragments, low-passage clinical isolates of varicella-zoster-virus (VZV), herpes simplex virus type I and II, several strains of adenoviruses, as well as...
on
were used. Noninfected human embryonic lung fibroblasts served
the gene of AD169, were synthesized
antibodies (MoAbs) directed against the early and immediate early
for detection of viral antigen expression.
monic lung fibroblasts after at least 4 weeks of culture. Monoclonal
lar assays were performed. To exclude
its of 28

Table 1. Patient Characteristics of 28 Patients Followed up
After BMT

<table>
<thead>
<tr>
<th>Marrow origin</th>
<th>No.</th>
<th>Seropositive pat.</th>
<th>Analysis before BMT (no. samples positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-identical unrelated donor</td>
<td>9</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>Mismatched family donor</td>
<td>3</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>HLA-identical sibling</td>
<td>16</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>GVHD prophylaxis</td>
<td>12</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>CSA</td>
<td>16</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>CSA/MTX</td>
<td>16</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td>16</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>Grade 0-II</td>
<td>12</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>Grade III-IV</td>
<td>11</td>
<td></td>
<td>BC</td>
</tr>
<tr>
<td>Symptomatic HCMV infections</td>
<td>11</td>
<td></td>
<td>BC</td>
</tr>
</tbody>
</table>

Abbreviations: CSA, cyclosporine; MTX, methotrexate.

as Epstein-Barr virus (EBV)-infected B-lymphoblastoid cell lines
were used. Noninfected human embryonic lung fibroblasts served
as negative control cells. Fibroblasts inoculated with the HCMV
strain AD169 were used as positive controls.

Viral DNA probes. For slot-blot and in-situ hybridization, the
labeled EcoR I DNA fragment of the HCMV AD169 (pCMS018, 11,067 bp, immediate early region) cloned in the bacterial vector
pACYC 184
was applied. Multiprimer labeling of this probe with
labeled EcoRI
viral DNA probes.

HCMV-DNA amplification, primer I, identical to position 1767 to
1786, and primer
complementary to the sense strand between
positions 1767 and 1913 of the fourth exon of the immediate early gene
of the HCMV strain AD169 using primer I and
was described. For
PCR.

Preparation of target DNA. DNA was extracted from urine (40
mL), blood cells, infected and noninfected fibroblasts, as well as
from tissue biopsies after proteinase K digestion using the phenol/
chloroform/soybean alcohol extraction and precipitation as de-
described.

Slot-blot hybridization. Two micrograms of each DNA prepara-
tion (200 µg/mL) or the total amount of DNA extracted from 20
mL urine were spotted onto prewetted nylon membranes (Biorad).
Prehybridization and hybridization of the membranes were performed as previously reported.

Autoradiography of the filters was performed on X-Omat AR
Kodak films at -80°C for 24 to 96 hours.

In-situ hybridization. Pretreatment of the glass slides, cryostat
sectioning of tissues, prehybridization, and hybridization were
performed as previously described. Autoradiography was per-
formed with NTB2 nuclear track emulsion (Eastman Kodak)
diluted 1:1 with 600 mmol/L ammonium acetate. After exposure
for 28 days at 4°C, slides were developed and evaluated with a Zeiss
interference contrast microscope. For each biopsy, hybridization-
picitive cells (25 grains over background) were counted in four
sections.

PCR. Amplification of a 147-bp DNA fragment between posi-
tions 1767 and 1913 of the fourth exon of the immediate early gene
of the HCMV strain AD169 using primer I and II has been
described. Briefly, 100 ng of extracted DNA (after two cycles of phenol/chloroform/soybean alcohol extraction and extrac-
tion and precipitation) were denatured at 94°C for 5 minutes and specifically
amplified in 50 µL reaction mixture of 10 mmol/L Tris-HCl, pH 9.6,
10 mmol/L MgCl2, 50 mmol/L NaCl, 1 mmol/L deoxyadenosine
triphosphate (dATP), 1 mmol/L dCTP, 1 mmol/L deoxyguanosine
triphosphate (dGTP), 1 mmol/L dTTP, 10 µg bovine serum albumin
(BSA), 0.25 µg of each primer I and II, as well as of 1 U
Ta polymerase (Perkin Elmer-Cetus, Emeryville, CA). Thirty-two
cycles, each of which included 3 minutes for annealing and primer
extension at 66°C followed by 1 minute of denaturation at 94°C,
were used.

To minimize the risk of contamination, the PCR technique was
physically separated from the laboratory area where DNA extrac-
tion and DNA recombinant research was performed. To exclude
the presence of polymerase inhibitors and to control the quality of
the extracted DNA, a fragment of the human HLA class I gene (4th

expression

Table 2. Detection of HCMV in Various Clinical Samples Using Culture, Slot-Blot and PCR Technique

<table>
<thead>
<tr>
<th>Pat. No.</th>
<th>Specimen</th>
<th>No. Samples</th>
<th>Detection of HCMV (no. samples positive)</th>
<th>Culture Positive</th>
<th>Slot-Blot Positive</th>
<th>PCR Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seronegative pat.</td>
<td>Analysis before BMT</td>
<td>4</td>
<td>Blood</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Analysis post-BMT</td>
<td>4</td>
<td>Blood</td>
<td>34</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Seropositive pat.</td>
<td>Analysis before BMT</td>
<td>24</td>
<td>Blood</td>
<td>30</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bone marrow</td>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Analysis post-BMT</td>
<td>24</td>
<td>Blood</td>
<td>273</td>
<td>29 (9)†</td>
<td>55 (12)†</td>
<td>148 (20)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>269</td>
<td>60 (16)†</td>
<td>42 (12)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bronch. lav.</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bone marrow</td>
<td>18</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

*Seronegative patients receiving their transplant and blood products from seronegative donors.
†Number of patients with at least one positive sample in the specimen analyzed and by the technique indicated.
EXON, 129-bp long) was amplified in all samples in parallel (unpublished results). If more than 400 ng DNA could be extracted from the material, analyzed amplification of the class I gene was performed in an extratube with 100 ng of the extracted DNA. If less than 400 ng DNA was precipitated from the material analyzed, the HLA class I gene control was amplified in the same tube (this only applies to some urine samples). Because of the thorough DNA extraction, applied amplification of the control DNA sequence could be demonstrated for all the samples analyzed. Each PCR analysis of clinical samples from patients before and after BMT included titered cloned pCM5018 and infected fibroblasts as positive, as well as noninfected fibroblasts, and at least four samples of DNA from peripheral blood of sero- and culture-negative normal donors as negative controls, to ascertain standardized conditions and specific amplification in each experiment. Three assays had to be discounted because of false positive negative controls. After thorough exclusion of contamination the tests were repeated and only evaluated when all the negative controls were negative for viral DNA amplification in the agarose gel as well as after hybridization. Results were considered valid only if consistent in at least two independent experiments. To control for false positive results because of lane carry-over in the Southern blot analysis, the order of the amplification products on the gels was randomly altered in repeated experiments.

**Analysis of HCMV amplification products.** Ten-microliter aliquots of each amplification reaction were electrophoretically separated in a 2% agarose gel and either stained directly by ethidium bromide or transferred by vacuum filtration onto nylon membranes (Biotrace; Biotest). After prehybridization (20 mmol/L phosphate-buffered saline [PBS], pH 7.0, 10X Magic Denhardt's, 7% sodium dodecyl sulfate (SDS), 5X SSC [20X SSC = 3 mol/L NaCl; 0.3 Na-citrate pH 7.0], salmon sperm DNA 100 μg/mL) for 1 hour at 50°C the filters were hybridized for Southern blot analysis with 1 X 10^6 cpm/mL of the gamma 32P-dATP-end-labeled internal oligonucleotide for 16 hours at 50°C. After two washings, each at 50°C for 1 hour, with 3X SSC, 10X Magic Denhardt's, 5% SDS in PBS first

**CMV—PCR**

![Figure 1](image-url)

**Fig 1.** Detection of HCMV-DNA by PCR technique. (A) The analysis of the amplified DNA sequence by evaluation of migration in agarose gel and ethidium bromide staining. The indicated amounts of the HCMV-DNA fragment pCM5018 were amplified. (B) An autoradiography showing a Southern analysis of the DNA fragments represented in (A). The fragments were transferred to nylon membrane and hybridization performed with a 32P-dATP–end-labeled internal oligonucleotide (exposure time, 4 hours). (C) An autoradiography demonstrating a Southern blot analysis of PCR products obtained after amplification of the HCMV-DNA–specific DNA sequence in DNA samples extracted from consecutive blood samples from a seropositive patient after allogeneic BMT. Hybridization was performed with a 32P-dATP–end-labeled internal oligonucleotide (exposure time, 12 hours). Lane 1, negative control (DNA sample extracted from the blood of a seronegative patient). Lanes 2 through 7, DNA extracted from consecutive blood samples (weeks 3 to 8 after BMT from a seropositive patient). Lane 8, marker pBR322/Hinfl.
and then with 1X SSC, 1% SDS, the filters were air-dried. Autoradiography of the filters was performed on an X-Omat AR Kodak film at −80°C.

RESULTS

Sensitivity and specificity of PCR analysis of HCMV. PCR analysis of the cloned titrated HCMV-DNA fragment pCM5018 showed that 10 fg of homologous HCMV-DNA amplified according to the previously described procedure was still directly visible after electrophoresis and ethidium bromide staining in a 2% agarose gel (Fig 1A). After blotting and hybridization with a 32P-end-labeled detection oligonucleotide (Fig 1B and C), a detection limit of 0.1 fg of amplified homologous HCMV-DNA corresponding to a sensitivity of about 10 virus copies could be achieved after 12 hours of exposure. Similar results were obtained in slot-blot hybridization assays of the amplification products. Reconstitution of the titrated cloned pCM5018 fragment with 1 μg of cellular DNA from noninfected fibroblasts did not reduce this sensitivity. Fifty different clinical isolates of HCMV showed equivalent amplification by PCR. In contrast, no amplified DNA fragments detectable on agarose gel-electrophoresis could be obtained from clinical isolates of other herpes or non-herpes viruses (VZV, herpes simplex virus [HSV] type I and II, EBV, several strains of adenoviruses). Similarly, control DNA extracted from noninfected fibroblasts gave no amplification signals even after hybridization with the specific detection oligonucleotides. However, fibroblasts inoculated with HCMV strain AD169 for 6 days were shown to be strongly positive by PCR.

PCR analysis of clinical samples after BMT. Four seropositive patients transplanted from seronegative donors who also received only seronegative blood products were followed-up after BMT for up to 10 weeks. None of the urine and blood samples obtained from these patients before and after BMT was found to be HCMV positive by PCR as shown in Table 2.

Twenty-four seropositive patients were investigated 10 days before and up to discharge from hospital or death after BMT for HCMV reactivation using the PCR technique in comparison with conventional culture and hybridization assays (Table 2). All 30 blood and 28 urine samples obtained before BMT were found to be negative for HCMV by culture and conventional slot-blot hybridization. However, after PCR amplification, peripheral blood cells of three IgG seropositive patients were shown to contain HCMV-DNA (Table 2). Two of these patients had been diagnosed as suffering from a primary or reactivated culture-proven HCMV infection 6 to 8 weeks before the HCMV-positive blood samples were drawn. The other patient had received multiple blood transfusions unscreened for HCMV before BMT. HCMV viremia was no longer demonstrable in these patients during the first 2 weeks after BMT.

Twenty of the 24 seropositive patients analyzed after BMT developed HCMV viremia and viruria 1 to 6 weeks posttransplantation, as shown by PCR amplification (Fig 1C and 2, Table 3). HCMV could be cultured from urine in 16 of these patients and from blood only in nine of the 20 PCR-positive cases. Slot-blot hybridization without DNA amplification showed the presence of HCMV in urine and blood samples of 12 patients. In nine of these patients, bone marrow cells were also found to harbor HCMV by PCR analysis. PCR analysis always showed HCMV detection in urine and blood before the other techniques showed presence of the virus (Fig 2 and Table 3). The results of the PCR and culture assay shown on Table 3 are representative for the group of patients with symptomatic (patient A) and asymptomatic infection (patient B). In two of the patients treated with ganciclovir and hyperimmunoglobulin, HCMV elimination from blood and also from other organ tissues could be documented. In eight other patients followed-up after BMT, clearance of the virus from the blood could also be documented by the PCR technique 6 to 18 months after BMT (data not shown). In one patient, the virus was still detectable in the blood 20 months after BMT.

The most sensitive detection of HCMV in lung and liver specimens of seropositive patients after BMT also could be achieved by the PCR technique when compared with other hybridization and cultural diagnostic assays (Table 4). All patients with interstitial pneumonia (N = 10), as well as one patient with obliterative bronchiolitis, were found by the PCR technique to carry HCMV in their lung tissues. Four seropositive patients with HCMV infection of blood or urine documented by culture assays and one seronegative patient were negative for HCMV in lung tissues as shown by all applied diagnostic test systems.

In 13 of 14 seropositive patients (seven included in the prospective analysis) with PCR-positive blood samples, HCMV could also be demonstrated by PCR technique in liver samples obtained postmortem or for diagnostic purposes. Ten of these patients had culture-proven viruria,
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Table 3. Comparison of Virus Culture Technique and PCR for HCMV Detection in Two Seropositive Patients After Allogeneic BMT

<table>
<thead>
<tr>
<th>Patient A (HCMV seropositive before BMT) with symptomatic HCMV infection</th>
<th>Patient B (HCMV seropositive before BMT) with asymptomatic HCMV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine</strong></td>
<td><strong>Urine</strong></td>
</tr>
<tr>
<td>Culture</td>
<td>Culture</td>
</tr>
<tr>
<td>PCR</td>
<td>PCR</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td><strong>Culture</strong></td>
</tr>
<tr>
<td>Culture</td>
<td>Culture</td>
</tr>
<tr>
<td>PCR</td>
<td>PCR</td>
</tr>
</tbody>
</table>

Table 4. Detection of HCMV in Organ Biopsy Specimens From Patients After BMT Using Culture, Slot-Blot, In-Situ Hybridization and PCR Technique

<table>
<thead>
<tr>
<th>Specimen</th>
<th>No. Samples</th>
<th>Culture Positive</th>
<th>Slot-Blot Positive</th>
<th>In-Situ Hybridization Positive</th>
<th>PCR Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>17</td>
<td>2</td>
<td>6</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Lung</td>
<td>16</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>11</td>
</tr>
</tbody>
</table>
EARLY DETECTION OF HCMV AFTER BMT

seropositive patients were found to develop HCMV infection before the occurrence of acute GVHD by PCR analysis only. Thus, at least a temporal association between HCMV infection and aGVHD could be shown for some patients analyzed. This result had not been possible applying conventional virus culture techniques. Furthermore, studies applying this very sensitive technique will have to evaluate the development of HCMV disease as well as for inducing or aggravating immunologic complications following BMT.

Because of its high sensitivity, the PCR technique presented might also allow improved evaluation of the different cells and organs affected by latent infection in healthy individuals, and thus help to further exclude blood products or transplants of risk for HCMV transmission.

REFERENCES


Early occurrence of human cytomegalovirus infection after bone marrow transplantation as demonstrated by the polymerase chain reaction technique

H Einsele, M Steidle, A Vallbracht, JG Saal, G Ehninger and CA Muller