Lymphocytopenia as an Unfavorable Prognostic Factor in Patients With Cytomegalovirus Infection After Bone Marrow Transplantation


Sixty-three recipients of an allogeneic marrow transplant were screened for the occurrence of cytomegalovirus (CMV) infection and clinical parameters possibly predicting the development of CMV disease in a retrospective study. Blood and urine samples obtained from these patients were screened weekly after bone marrow transplantation (BMT) for the presence of CMV by polymerase chain reaction (PCR) and virus culture technique. Forty-six of the 63 patients studied were found to be CMV-positive by PCR technique in blood and urine samples at a median of 29 days after BMT. In 33 of these 46 patients, CMV could be cultured from urine samples and 16 of the 46 had culture-positive viremia. Twenty-eight of these 46 PCR-positive patients developed CMV disease. Whereas PCR assays showed an optimal negative predictive value and sensitivity for the development of CMV disease, their positive predictive value was 61% and could not be remarkably increased when culture-positive viremia (64%) and viremia (69%) were considered. Acute graft-versus-host disease (GVHD) grade 2 to 4 (P < .05), but not underlying disease, conditioning therapy, or GVHD prophylaxis, was associated with CMV infection. On day + 49, a remarkable decrease (P < .001) in the lymphocyte count, as well as in the absolute number of CD4+, CD8+, and CD56+ lymphocytes, occurred only among the patients who later developed CMV disease. The decrease of all of these cell counts, but predominantly the CD4+ T cells, to less than 100/µL on day + 49 after BMT showed a very high positive predictive value (100%) for the development of CMV disease in patients with PCR-positive viremia. Persisting CD4 lymphopenia after antiviral therapy was only observed in patients who finally died of CMV disease. Thus, immunophenotyping of the patients after BMT in addition to a highly sensitive virus detection assay might help to identify patients at high risk to develop CMV disease and indicate the need for additional adoptive immunotherapy.

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MATERIALS AND METHODS

Patients. Characteristics of 63 consecutive patients undergoing allogeneic BMT at our institution between 1989 and 1991 and analyzed in this study are shown in Table 1. For conditioning regimen, total body irradiation (TBI) was performed on 3 successive days (2 Gy twice a day, lung shielding at 10 Gy). Cyclophosphamide 2 × 60 mg/kg was infused following TBI (Cy/TBI). All but one patient with severe aplastic anemia (SAA) received cyclophosphamide in a dose of 50 mg/kg on 4 successive days (Cy). In patients with accelerated chronic myelogenous leukemia (CML) or acute leukemia not in first remission, etoposide (VP-16/Cy/TBI) was added in a dose of 30 mg/kg on day -3. All of the patients studied received a marrow transplant from a serologically HLA-matched donor (53 from a sibling, 10 from an unrelated donor). Seropositive patients, as well as those receiving a marrow graft from a seropositive donor, received blood products unscreened for CMV and a CMV hyperimmunoglobulin prophylaxis. Except for three cases, all of the seronegative patients receiving a seronegative graft were transfused with blood products from seronegative blood donors. Oral acyclovir was administered at a dosage of 4 × 400 mg/d for prophylaxis of herpes simplex virus infections until day +100 after BMT. Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Monitoring for CMV infection. All of the patients in this study were monitored weekly by virus culture and PCR technique for the development of CMV viremia or viruria after BMT. CMV infection was diagnosed when blood and urine samples were positive for...
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| Three groups of patients were differentiated according to CMV infection and development of CMV disease. 

Abbreviations: AML, acute myelogenous leukemia; CR, complete remission; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; c.P., chronic phase; a.P./b.c., accelerated phase/blast crisis; CSA, cyclosporin; MTX, methotrexate.

CMV DNA by PCR technique with and without culture-proven viremia and/or viruria.

CMV disease. Patients were diagnosed with symptomatic CMV infection when CMV infection detected by PCR was associated with organ dysfunction (interstitial pneumonia, enteritis, and hepatitis). Asymptomatic CMV infection was diagnosed if CMV infection was confirmed in two PCR assays of consecutive blood samples, but no organ dysfunction could be demonstrated. CMV pneumonia was diagnosed on the basis of dyspnea, interstitial infiltrates on chest radiograph, and a positive CMV culture of bronchial washings. Diagnosis of CMV enteritis was based on clinical and endoscopic signs of enteritis and colitis plus histologic demonstration of CMV inclusions, positive in-situ hybridization assays, and positive culture for CMV from the tissue samples obtained by biopsy. The diagnosis of CMV hepatitis was based on abnormal liver function tests occurring in association with culture-proven CMV viremia. Postmortem analysis of 10 patients with the diagnosis of CMV disease confirmed the diagnosis of CMV-interstitial pneumonia (CMV-IP) in nine patients and CMV-hepatitis in one patient.

All 28 patients with CMV disease (interstitial pneumonia, enteritis, hepatitis) received antiviral therapy (2 × 5 mg/kg ganciclovir for 14 days and CMV-hyperimmunoglobulin infusions for 5 consecutive days) when culture-proven viremia was detected. Therapy was initiated in all patients later than day 52 after BMT (median, 65; range, 53 to 80 days after BMT). Despite receiving antiviral therapy, 11 of 20 patients with CMV-IP died of CMV disease after BMT.

Virus culture. Blood and urine were conventionally inoculated on human embryonic lung fibroblasts maintained according to standard tissue culture techniques. CMV was identified by production of its characteristic cytopathic effects on human embryonic lung fibroblasts after at least 4 weeks of culture. Monoclonal antibodies directed against the early and immediate early antigen were additionally applied 48 hours after inoculation of the fibroblasts for detection of viral antigen expression.

PCR technique. Amplification of a 147-bp DNA fragment between positions 1767 and 1913 of the fourth exon of the immediate early gene of the CMV strain AD169 using primer I and II has been described before. Briefly, 100 ng of extracted DNA or the total amount of DNA extracted from 10 mL urine was denatured at 94°C for 5 minutes and specifically amplified. 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 administered. To minimize the risk of contamination, the PCR technique was physically separated from DNA extraction and precipitation. To exclude the presence of polymerase inhibitors and to test the quality of the extracted DNA, a DNA fragment of the human HLA class I genes (fourth exon, 129-bp long) was amplified in all samples in parallel. Results were considered valid only if consistent in at least two independent experiments and when all the negative controls did not show amplification in either the agarose gel or after hybridization. Detection of the amplification products in a 2% agarose gel or by Southern blot analysis with gamma-32P-d-ATP-end-labeled internal oligonucleotide has been described previously.

Indirect immunofluorescence analysis. After the analysis of the differential blood count of the first consecutive 26 patients demonstrated a correlation between a decrease in the lymphocyte count and symptomatic CMV infection, the following consecutive 37 patients were additionally studied for the absolute numbers of CD4+, CD8+, and CD56+ lymphocytes. Thus, immunophenotyping of the peripheral blood mononuclear cells was performed in 11 patients with asymptomatic, 13 patients with symptomatic, and 13 patients without CMV infection. Mononuclear cells obtained by Ficoll Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation from heparinized peripheral blood were labeled by the indirect immunofluorescence technique with the use of monoclonal antibodies (MoAbs) as first and F(ab')2 fragments of fluorescein-isothiocyanate (FITC)-conjugated goat antimouse IgG as second antibody layer. Fluorescence was evaluated on a FACSS IV cell sorter (Becton Dickinson, Sunnyvale, CA). Dual-scatter gates (forward × 90° scatter) were set to electronically select subpopulations of which the fluorescence distribution was analyzed. The green fluorescence of the FITC-labeled cells was measured through a 530-nm band pass filter. After proper compensation with control beads, the fluorescence intensities (log scale, 4 decades) and the scatter signals (linear scale) were analyzed by a data lister and evaluated digitized on an IBM-AT (Sindelfingen, Germany) using in-house programs.

MoAbs. For immunophenotyping, the following MoAbs were used routinely: WT31 (α-β-T-cell receptor), Leu-19 (CD56), CALLA (CD10), and Leu-12 (CD19), which were purchased from Becton Dickinson; My7 (CD3) and B1 (CD20) were derived from Coulter Immunology (Hialeah, FL); OKT 10 (CD38), OKT 4 (CD1), OKT 11 (CD2), OKT 3 (CD3) were purchased from Ortho Diagnos-
tics (Raritan, NJ), TT1 (CD4) and TT2 (CD8),

17 Tü1 (CD5), TÜ93 (CD7),

17 TM1 (CD14),

17 and TÜ69 (CD25),

18 as well as the HLA-class II antigen specific antibodies TÜ22 (anti-DQ common),

TÜ36 (anti-DR common), TÜ39 (anti-DR, DP, DY),

18,19 and FA (anti-DP),

20 were derived from our laboratory.

Statistical analysis. In a retrospective test, several parameters were screened for their importance as prognostic factors predicting CMV disease. Tests performed in this context are of explorative nature. All tests were determined two-sided for \( \alpha = 0.05 \).

In the analysis of differences of absolute numbers of patients or positive results either of the PCR technique or the culture assay, chi-square analysis was used. Probability values of less than 0.05 were considered significant. The cell counts of each individual patient at each time point given were compared for the three patient groups by the Wilcoxon-Mann-Whitney U-test.

RESULTS

Diagnosis of CMV infection. CMV infection occurred predominantly among seropositive donors or patients receiving marrow transplants from seropositive patients (Table 1). Three seronegative patients with seronegative marrow donor developed CMV infection after having received multiple blood products from random, CMV-un-screened blood-bank donors for severe gastrointestinal bleeding.

CMV excretion in the urine could be diagnosed in 46 of 63 patients analyzed as early as 25 days after BMT (median; range, 0 to 38 days) using the PCR technique. In all of these 46 patients, PCR technique showed the presence of the virus also in urine and blood samples at a median of 29 days after BMT (range, 0 to 42 days). By virus culture technique, viruria could be demonstrated in 33 patients at a median of 40 (range, 8 to 70) days and viremia in 16 patients at a median of 45 (range, 8 to 78) days after BMT. CMV excretion in the urine was found to precede viremia as demonstrated by both techniques. Only 12 of 33 patients with culture-proven viruria were found to be viremic by culture technique. In all of the patients with positive culture from at least one site, the virus could be detected in blood and urine by PCR technique. In 4 of 9 patients with positive PCR, but negative culture assay for CMV in blood and urine, the virus could be grown from the organ affected during symptomatic CMV infection or postmortem.

Correlation of different diagnostic and clinical parameters with the development of CMV disease. Only 28 of 46 PCR-positive patients developed CMV disease. Twenty of them suffered from CMV-induced interstitial pneumonia, six from CMV-enteritis, and two from CMV-hepatitis. Symptoms of CMV disease occurred at a median of 56 days after BMT (range, 49 to 63 days).

None of 27 patients who had a negative PCR assay went on to develop CMV disease. However, 17 patients with negative culture assay from all of the blood samples and seven patients with negative culture assay from all of the urine samples analyzed later developed CMV disease. Although the specificity of culture-proven viremia for the development of CMV disease was higher than for culture-proven viruria or a positive PCR assay, the positive predictive values of culture-proven viremia and viruria for the development of CMV disease were only slightly higher than the positive predictive value of PCR-positive blood and urine samples (Table 2). However, sensitivity and negative predictive values of the PCR assay were found to be 100% in contrast to the much lower sensitivity and negative predictive value of culture-proven viremia and viruria.

Only a small number (n = 5) of patients were found to suffer from CMV infection by PCR technique within the first 10 days after BMT—two patients on the day of marrow transplantation. None of these patients developed CMV disease.

The incidence of grade 2 to 4 acute graft-versus-host disease (GVHD) was significantly lower (\( P < .05 \)) among the patients who did not develop CMV infection compared with those with CMV infection (Table 1). However, the percentage of cases with severe acute GVHD did not differ between the patients with asymptomatic (61%) and symptomatic (82%) CMV infection (\( P > .05 \)). In contrast to male patients, females, who were predominantly seronegative before BMT, were more frequent in the group without CMV infection. No remarkable difference could be found between the patients without and with asymptomatic or symptomatic CMV infection for age, GVHD prophylaxis, and conditioning regimen (\( P > .05 \)).

Reconstitution of the peripheral blood leukocytes and lymphocytes. Analysis of the leukocyte counts at weekly intervals after BMT showed no remarkable difference between the patients with symptomatic and those with asymptomatic or without CMV infection (\( P > .05, P > .05 \)) until day +56. On day +63 post-BMT, a remarkable decrease in the leukocyte count could be observed only in the patients with symptomatic CMV infection (\( P < .001 \)). Total lymphocyte counts evaluated morphologically in the 63 cases analyzed were not different comparing the patients without and with asymptomatic CMV infection (Fig 1). Whereas on day +55 after BMT the median number of lymphocytes was not different among the three groups of patients (\( P > .05 \)), in

| Table 2. Sensitivity, Specificity, and Predictive Values of PCR or Culture-Proven CMV Viruria and/or Viremia for the Development of CMV Disease in 63 Marrow Transplant Recipients |
|---------------------------------|--------------------|------------------|
| CMV Detection By | CMV Disease (n = 28) | No CMV Disease (n = 35) |
| PCR assay | | |
| Positive* | 28 | 18 |
| Negative† | 0 | 17 |
| Culture assay | | |
| Viremia | 11 | 5 |
| Sensitivity, 100% | 39% |
| Specificity, 49% | 86% |
| Positive predictive value, 61% | 69% |
| No viremia | 17 | 30 |
| Negative predictive value, 64% | 66% |
| Viruria | 21 | 12 |
| Sensitivity, 75% | 66% |
| Specificity, 66% | 64% |
| Positive predictive value, 64% | 77% |
| No virea | 7 | 23 |
| Negative predictive value, 77% |

* Detection of CMV-DNA in blood, as well as in urine samples.
† Negative for CMV-DNA in blood, as well as in urine samples.
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patients with symptomatic infection, the lymphocyte count was found to be remarkably lower on day +49 post-BMT compared with the two other groups (Fig 1, P < .001).

FACS-analysis of the peripheral blood mononuclear cells performed weekly in 37 of 63 patients, analyzed before the development of CMV disease or antiviral therapy, showed a remarkable decrease in the absolute numbers of the CD56+, CD8+, and CD4+ T lymphocytes (Fig 2) on day +49 after BMT, particularly in the group of patients with symptomatic CMV infection (P < .001 for CD4+, CD8+, and CD56+ lymphocytes). The cell counts of the lymphocyte subpopulations continued to decrease until the occurrence of CMV disease.

In 8 of 13 patients with symptomatic CMV infection who could be analyzed, a concomitant lower expression of MHC class II antigens and of the interleukin (IL)-2 receptor (CD25) on CD7+ lymphocytes was observed from day 49 until day 63 after BMT. For patients with a positive PCR signal, high positive and negative predictive values for the development of CMV disease could be shown for the PCR assay. The positive predictive value for the development of CMV disease was not very high for the PCR assay (61%) and unfortunately could not be remarkably increased when culture-proven viruria (64%) and/or viremia (69%) were considered. Sensitivity, specificity, and positive predictive value of culture-proven viremia were comparable with the values recently published. Sensitivity, specificity, and predictive values of CMV viruria.

number of CD8+ T lymphocyte or CD56+ natural killer cells could be found during the course of the infection (P > .05). In contrast, persisting CD4+ T lymphocytopenia was only observed among patients with fatal CMV infection. In patients who survived CMV disease, CD4+ T cells recovered after day +63 (Fig 3, P < .01).

DISCUSSION

The PCR technique, as previously shown and confirmed in this study for a larger cohort of patients, allows CMV detection in blood and urine samples significantly earlier after BMT than the virus culture technique. As demonstrated previously, in several patients with CMV disease, the virus could not be cultured from blood and urine samples before the onset of symptoms. In contrast, as shown in this study, PCR technique allowed diagnosis of CMV infection in all patients at a median of 31 days before the occurrence of CMV disease. Also, an optimal sensitivity and negative predictive value for the development of CMV disease could be shown for the PCR assay. The positive predictive value for the development of CMV disease was not very high for the PCR assay (61%) and unfortunately could not be remarkably increased when culture-proven viruria (64%) and/or viremia (69%) were considered. Sensitivity, specificity, and positive predictive value of culture-proven viremia were comparable with the values recently published. Sensitivity, specificity, and predictive values of CMV viruria.

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In 8 of 13 patients with symptomatic CMV infection who could be analyzed, a concomitant lower expression of MHC class II antigens and of the interleukin (IL)-2 receptor (CD25) on CD7+ lymphocytes was observed from day 49 until day 63 after BMT. For patients with a positive PCR signal, high positive and negative predictive values for the development of CMV disease could be demonstrated for a decrease in the lymphocyte count to less than 300/μL and a decrease in the CD4+ and CD8+ T cells, as well as CD56+ lymphocytes, to less than 100/μL on day +49 after BMT (Table 3). An optimal sensitivity and positive predictive value of 100% could be shown for the CD4+ T-cell count.

When patients with lethal CMV disease were compared with those with nonlethal CMV disease, no remarkable difference in the total leukocyte or lymphocyte count, or in the
Morphologically defined the fact that patients receiving the transplant from an unre-
munophenotyping of 24 CMV infection were compared with those with asymptom-
tic CMV infection. None of the other disease- or therapy-
ated donor were included in the study. But, at least in this study, the percentage of patients with severe acute GVHD 
occurred among the patients who later developed CMV disease. In contrast, on day +49 after BMT, a significant decline of the lymphocyte count occurred among the patients who developed CMV dis-
ease. Immunophenotyping of the peripheral blood mononuclear cells of 37 patients showed a remarkable decrease in the natural killer and CD8+ T cells, but predominantly in the CD4+ T lymphocytes on day +49 after BMT. High predictive values for the development of CMV disease in patients with PCR-proven CMV infection could be shown for a decrease in the absolute numbers of these lymphocyte 
subpopulations, especially for a decrease of the CD4+ T cells to less than 100/μL on day +49.

Thus, in contrast to previous studies based on positive culture from blood and urine samples or bronchoalveolar lavage, PCR assays with an optimal negative predictive value and sensitivity allowed the diagnosis of CMV infec-
tion in all the patients who later developed CMV disease. According to our results, preemptive antiviral therapy based on a highly sensitive technique as, for example, PCR assays, should be recommended for all patients at high risk to de-
velop CMV infection following allogeneic BMT, such as patients with severe acute GVHD or patients who have un-
were slightly higher in this study when compared with the ones from the Seattle study. Persistent asymptomatic CMV viremia after allogeneic BMT had been already described.

In agreement with animal studies and clinical observations, CMV infection was associated with the occurrence of severe acute GVHD (grade 2 to 4). The high incidence of acute GVHD and also of CMV-IP might be due to the fact that patients receiving the transplant from an unrelated donor were included in the study. But, at least in this study, the percentage of patients with severe acute GVHD did not differ significantly when patients with symptomatic CMV infection were compared with those with asymptomatic CMV infection. None of the other disease- or therapy-related parameters analyzed correlated with the occurrence of CMV infection or disease.

According to PCR analysis of blood and urine samples and in correlation to the clinical presentation, three groups of the 63 patients analyzed were distinguished: 17 patients did not develop CMV infection, 18 patients presented with asymptomatic, and 28 with symptomatic CMV infection. Only patients with symptomatic CMV infection showed a decrease in the leukocyte count on day +63 after BMT—at the time of or after the onset of CMV disease (median, day +56 after BMT). Therefore, the decrease in the leukocyte count was not found to be suitable as a predictive factor for the development of CMV disease. In contrast, on day +49 after BMT, a significant decline of the lymphocyte count occurred among the patients who later developed CMV disease. Immunophenotyping of the peripheral blood mononuclear cells of 37 patients showed a remarkable decrease in the natural killer and CD8+ T cells, but predominantly in the CD4+ T lymphocytes on day +49 after BMT. High predictive values for the development of CMV disease in patients with PCR-proven CMV infection could be shown for a decrease in the absolute numbers of these lymphocyte 
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Lymphocytopenia predicts CMV disease after BMT

Doherty transplantation from an HLA-mismatched or unrelated donor. Treating such patient groups after two consecutive positive PCR signals with ganciclovir and CMV hyperimmune globulin, we observed a significant decrease in the incidence of CMV disease in a preliminary study (unpublished data). To avoid overtreatment in PCR-positive marrow transplant recipients not belonging to these risk groups—considering the only moderate predictive value of PCR assays for the development of CMV disease—one could monitor effectively these patients without initiating therapy for a decrease in the lymphocyte count, especially the total number of CD4+ T cells. In case of a decrease in these cell counts, antiviral therapy should be started immediately.

Despite the introduction of antiviral therapy, some patients, mostly ones lacking anti-CMV-specific cytotoxic T lymphocytes, do not respond to treatment and die of CMV disease. Opposite to the increase in natural killer and CD8+ T lymphocytes approximately 2 weeks after culture-proven CMV infection, the number of CD4+ T lymphocytes remained low among the patients with lethal CMV infection in contrast to the patients with nonlethal CMV disease. Symptoms of CMV disease developed a median of 41 days after the occurrence of acute GVHD and at a time when in all of the patients studied, hematologic and immune reconstitution had been at least partially achieved. The decrease in the number of leukocytes indicates a CMV-mediated myelosuppression only in patients with symptomatic CMV infection. Myelosuppression has been suggested to result from infection of marrow stroma cells leading to a reduced granulocyte colony-stimulating factor (G-CSF) level, but increased gamma interferon and tumor necrosis factor-alpha production by mononuclear cells in the presence of CMV-infected marrow fibroblasts, as well as a direct inhibition of growth and differentiation of progenitor cells. The myelosuppressive side effect of ganciclovir treatment might have aggravated the observed decrease in the leukocyte count in the patients with CMV disease.

However, the earlier and remarkable decrease in the number of lymphocytes, especially CD4+ T cells in patients with CMV disease before antiviral therapy was started, appeared to be due to a preceding specific CMV-related disturbance of T-cell regeneration. The decrease in the MHC class II and CD25 expression on CD7+ T lymphocytes in these patients seemed to support an additional CMV-mediated defect in T-cell activation.

Immunocytologic and in-situ hybridization analysis showed CMV to infect all lymphocyte subpopulations, but predominantly the CD4+ T cells. Growth inhibition or direct lysis by a selective infection of CD4+ T lymphocytes, as well as a virus-mediated alteration of the cytokine cascade, might participate in the induction of severe CD4+ T lymphopenia in patients with symptomatic CMV infection.

Prospective studies will have to address the predictive relevance of CD4+ lymphocytopenia in patients with CMV infection after BMT when treated with ganciclovir. Patients with CMV infection and persisting severe CD4 lymphopenia may need additional immunostimulation to augment antiviral immune responses for better control of CMV disease. Early application of cytokines or adoptive immune transfer of donor lymphocytes might improve the prognosis of these patients with often fatal CMV disease.

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References


Lymphocytopenia as an unfavorable prognostic factor in patients with cytomegalovirus infection after bone marrow transplantation

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