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Early Detection of Human Cytomegalovirus Viremia in Bone Marrow Transplant Recipients by DNA Amplification

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Surveillance blood cultures for human cytomegalovirus (HCMV) are commonly used to identify the bone marrow transplant (BMT) recipients with the highest risk of serious HCMV disease and for whom early interventional ganciclovir therapy would be beneficial. We monitored 36 allogeneic BMT recipients weekly for the presence of HCMV in the blood from 0 to 100 days posttransplantation. Viable HCMV in leukocytes (WBC) was detected by shell vial and tube culture methods. HCMV DNA in WBC and plasma was detected by PCR and DNA hybridization using primers and a probe from the *Eco*RI fragment D region of HCMV AD169. A uracil-*N*-glycosylase-dUTP PCR protocol was used to prevent false-positive results due to amplicon carryover. Seventeen patients had multiple consecutive positive samples containing HCMV DNA in plasma or WBC. In 14 of 17 patients, HCMV was also detected by blood culture. HCMV DNA was detected sporadically in six patients, none of whom had positive cultures. One patient had HCMV viremia detected by WBC culture only. The remaining 12 patients had no positive PCR assays or blood cultures. For the patients with positive blood cultures, PCR detection of HCMV DNA in plasma preceded detection of HCMV in culture by a mean of 8 days and detection in WBC preceded detection in culture by 6 days. HCMV disease (interstitial pneumonia) was documented for two patients with viremia (blood culture and PCR positive) and one patient without viremia (blood culture and PCR negative). The earlier recognition of high-risk patients provided by detection of HCMV DNA in plasma or WBC may improve the efficacy of early interventional antiviral therapy.

Human cytomegalovirus (HCMV) is a significant cause of morbidity and mortality in solid-organ transplant and bone marrow transplant (BMT) recipients (8, 16, 25, 27). Although the virus can be isolated from a variety of different specimens, demonstration of viremia is generally considered to have the best correlation with clinically significant infection (16, 17). Optimal detection of HCMV viremia by culture requires isolation of peripheral blood leukocytes (WBC) by gradient centrifugation and the use of both shell vial and conventional tube culture methods (9, 11).

The availability of specific antiviral therapy for HCMV infections has created a need for rapid and specific tests for monitoring patients at risk for developing disease (22). Detection of HCMV immediate early antigen by immunohistochemistry (2, 24) and detection of HCMV DNA (6, 12, 14) or mRNA (1) in WBC are promising approaches to the problem. Recently, HCMV DNA has been detected in sera from congenitally infected infants and from renal transplant recipients with active HCMV infections (3), in plasma from AIDS patients with acute visceral disease (23), and in sera from BMT recipients and patients with leukemia who had HCMV pneumonia (13, 28). These studies suggest that the presence of HCMV DNA in cell-free fractions of the blood correlates well with the onset of visceral disease.

Two approaches are currently employed to prevent HCMV disease in seropositive BMT recipients. One approach involves the use of either acyclovir or ganciclovir given prophylactically to all patients (10, 18, 26). The other approach involves early or preemptive use of ganciclovir in patients with HCMV detected in bronchoalveolar lavage fluid, blood, urine, or throat specimens (19, 22). Although there is no consensus on the relative merits of prophylaxis versus early interventional therapy, early intervention based on HCMV surveillance culture results has the advantage of sparing a sizeable number of patients not destined to develop disease from the cost and toxicity of ganciclovir therapy. The success rate for early interventional therapy may be improved if the patients at highest risk for developing HCMV disease could be identified earlier.

Surveillance blood cultures for HCMV are used routinely at Emory Clinic to identify those allogeneic BMT recipients with the highest risk of developing serious HCMV disease. Patients with positive blood cultures are treated preemptively with ganciclovir. In this study, we compared conventional virological blood culture methods with a PCR-based assay for detection of HCMV DNA in both WBC and plasma samples obtained from 36 allogeneic BMT recipients. The patients were monitored weekly for HCMV viremia from 0 to 100 days posttransplantation. We found that detection of HCMV DNA in plasma or WBC is more sensitive than culture for detection of viremia and provides for earlier recognition of these patients at highest risk for HCMV disease.

(This work was presented in part at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, La., 17 to 20 October 1993 [20]).

MATERIALS AND METHODS

Patients and specimens. Blood samples were collected from 36 allogeneic BMT recipients once a week from 0 to 100 days posttransplantation. Twenty-one patients were HCMV seropositive before transplantation, and 3 of the 15 initially seronegative patients received bone marrow from a seropositive donor. The lithium-heparin anticoagulated whole blood was delivered promptly to the laboratory. WBC were recovered from 3.5 ml of blood by a single-step centrifugal technique (Polymorphprep; Nycomed, Oslo, Norway). The mononuclear and polymorphonuclear cell bands were removed from the gradient, pooled, and

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TABLE 1. Correlation of HCMV PCR and WBC culture results for 36 allogeneic BMT recipients

WBC culture result	No. of patients with indicated PCR result			
	WBC		Plasma	
	Positive	Negative	Positive	Negative
Positive	13	2	14	1
Negative	6	15	8	13

washed twice in tissue culture medium. The washed WBC were used to inoculate shell vials and cell culture tubes, and an aliquot was frozen at −80°C for PCR. The number of WBC in each sample was not determined. Plasma obtained from the same blood specimens was also frozen at −80°C for PCR.

Clinical and laboratory data for all patients were compiled by a research nurse. The patients were evaluated for increased liver function tests, graft-versus-host disease, rash, enteritis, hemorrhagic cystitis, retinitis, interstitial pneumonia, antiviral history, WBC count, and HCMV culture results. The research nurse and the clinicians caring for the patients were blinded to the HCMV PCR results.

Blood cultures. A 200-μl volume of the washed WBC was inoculated into each of three shell vials containing MRC-5 cells, and 300 μl was inoculated into each of two human foreskin fibroblast roller tube cultures. The shell vials were stained after 36 to 72 h of incubation in an indirect immunofluorescence assay (DuPont, Doraville, Ga.) to detect HCMV infection of the monolayers. The roller tube cultures were examined for a total of 21 days for the appearance of characteristic cytopathic effects.

PCR. Frozen 100-μl aliquots of WBC and plasma were thawed and heated at 95°C for 10 min. Plasma was diluted 1:1 with a Tris-Triton-EDTA buffer (10 mM Tris [pH 8.0]), 1% Triton X-100, 1 mM EDTA) prior to being heated. A 10-μl sample of these crude lysates was added directly to the PCR cocktail.

PCR was carried out by the method of Hsia et al. (12), with minor modifications. The primer pair used (primers 459 and 627) amplifies a 152-bp band from *Eco*RI fragment D of the HCMV genome. All reactions were hot-started to decrease nonspecific amplification. False-positive results due to amplicon cross-contamination were controlled by standard practices (19) and the use of dUTP and uracil-*N*-glycosylase (15).

A low positive control and a reagent blank were included in each run. A second amplification reaction using primers (PCO4 and GH20) that amplify a segment of the human β-globin gene was performed for each specimen for which the WBC culture was positive and PCR results were negative and for specimens for which there were discrepancies between the WBC and plasma PCR results (21). This was done to detect the presence of PCR inhibitors.

Amplified DNA was separated by agarose gel electrophoresis, and the 152-bp band was visualized by ethidium bromide staining and UV light transillumination. The DNA was then transferred to nylon membranes by Southern blotting. Detection of specific probe hybridization on Southern blots was accomplished with biotin-labeled internal probe 628 (12) and an avidin-alkaline phosphatase, chemiluminescent substrate detection system (Southern Lights; Tropix, Bedford, Mass.). Blots were imaged on X-ray film. The PCR assay reliably detects 0.01 pg of purified DNA from HCMV strain AD169. Purified HCMV DNA was kindly provided by P. Pellet, Centers for Disease Control and Prevention, Atlanta, Ga.

RESULTS

HCMV was detected by WBC culture in 15 patients (42%) and in 22 of 371 specimens (6%). Sixteen of the positive cultures were detected by the shell vial technique, and six required conventional tube cultures and extended incubation for detection of the virus. The average time for detection of a positive shell vial was 2.8 days, and that for conventional tube cultures was 13.8 days.

PCR of WBC detected HCMV DNA in 19 patients (53%) and in 74 of 371 specimens (20%). In 68% of these patients, HCMV was also detected by WBC cultures. PCR of plasma detected HCMV DNA in 22 patients (61%) and in 110 of 368 specimens (30%). WBC cultures were also positive for 64% of these patients. Southern blotting and hybridization with the labeled probe were required to detect 30% of the PCR-positive specimens regardless of the specimen type.

The correlation of PCR and culture for detection of HCMV in the blood is shown in Table 1. With WBC culture used as the "gold standard", the sensitivity and specificity of WBC PCR were 87 and 71%, respectively. The sensitivity and specificity of plasma PCR were 93 and 62%, respectively. For 16 patients, HCMV DNA was detected in multiple consecutive WBC samples, and 13 of these patients had at least one positive WBC culture. HCMV DNA was detected in multiple consecutive plasma samples from 17 patients, and viremia was found in 14 of these patients by culture. There were six patients who had HCMV DNA detected sporadically in plasma and WBC (two patients), plasma only (three patients), or WBC only (one patient). None of these patients had positive WBC cultures, and only one of the patients was positive when the PCR assay was repeated. One patient had HCMV viremia documented 13 days posttransplantation by culture only. Twelve patients had no HCMV DNA detected in WBC or in plasma and no positive WBC cultures at any point during the study period.

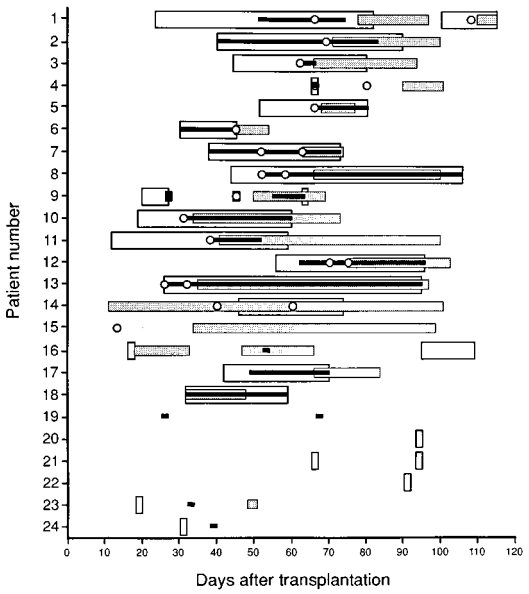

FIG. 1. Schematic representation of the temporal relationship of the detection of circulating DNA, viremia, and ganciclovir treatment for 24 BMT recipients from whom at least one sample was positive for HCMV. Open circles, positive WBC cultures; solid lines, positive interval WBC PCR; open bars, positive interval plasma PCR; shaded bars, ganciclovir treatment.

The mean times to onset of viremia as detected by WBC culture, WBC PCR, and plasma PCR were 49, 43, and 41 days posttransplantation, respectively. For patients with positive WBC cultures, detection of onset of viremia by plasma PCR preceded that by culture by a mean of 8 days, and detection by WBC PCR preceded detection by culture by 6 days. The temporal relationship of the detection of circulating DNA, viremia, and ganciclovir therapy for the 24 patients who had at least one sample positive for HCMV is represented schematically in Fig. 1.

Preemptive ganciclovir therapy was initiated in 15 patients in response to positive WBC cultures. An additional seven patients received ganciclovir because of a high index of clinical suspicion of HCMV disease. Although the physicians caring for the patients were not aware of the PCR results, ganciclovir was given to all patients with multiple consecutive blood samples containing HCMV DNA regardless of the WBC culture results.

Objective evidence for HCMV disease was found in only three patients. All three had HCMV interstitial pneumonia documented by hypoxia, interstitial infiltrates, a positive culture of bronchoalveolar lavage fluid, and absence of other pathogens. In two patients, viremia preceded the development

of pneumonia and was documented by both culture and PCR. For the remaining patient, antecedent viremia was not detected by culture or PCR.

HCMV DNA persisted in plasma or WBC in five patients at the end of ganciclovir therapy. One patient also had a positive blood culture for HCMV at the end of treatment. None of the three patients thought to have developed HCMV interstitial pneumonia had detectable HCMV DNA in the blood after ganciclovir therapy.

DISCUSSION

The aim of this study was to compare conventional virological techniques with a PCR-based assay for detection of HCMV in blood samples used to monitor BMT recipients. Weekly surveillance blood cultures for HCMV are done routinely in our hospital to identify the BMT recipients with the highest risk of developing severe HCMV infections and for whom preemptive ganciclovir treatment would be beneficial. On the basis of the PCR results, the patients fell into three groups: patients with viral DNA detected in multiple consecutive samples, patients with viral DNA detected sporadically in blood, and patients with no viral DNA detected during the first 100 days posttransplantation.

We found that the onset of viremia was preceded by the presence of HCMV DNA in multiple consecutive WBC or plasma samples for 14 of 15 patients. The remaining patient had a positive WBC culture 13 days posttransplantation, but no DNA in WBC or plasma was detected at any time. The virus was recovered only from tube cultures after 3 weeks of incubation, indicating that the quantity of virus was small and that the false-negative PCR results were probably due to sampling error. HCMV was not isolated from any other body site or fluid sample from this patient during the study.

In the patients with both positive blood cultures and positive PCR results, detection of the onset of viremia by PCR of either WBC or plasma preceded detection by culture by 6 to 8 days on average. For two of the patients who developed HCMV pneumonia, viremia was documented approximately 2 weeks earlier by PCR. Although the physicians were not aware of the PCR results, ganciclovir was given to all patients with multiple consecutive blood samples containing HCMV DNA, including three patients with negative WBC cultures. The low incidence of HCMV pneumonia in our study patients was probably due to the preemptive use of ganciclovir in patients with WBC cultures positive for HCMV. Earlier recognition of high-risk patients through the use of PCR methods may improve the effectiveness of preemptive ganciclovir therapy and prevent the development of HCMV pneumonia in some patients.

There were six patients in whom HCMV DNA was detected sporadically during the study period. For five of these patients, the results are probably due to DNA cross-contamination of PCR assays. Only one patient was positive on repeat, and that patient received ganciclovir because of a high index of clinical suspicion (negative WBC culture). The false-positive rate for the PCR assays was 1.3% (10 of 739 assays). The source of the sporadic false-positive reactions was probably template cross-contamination rather than amplified-product contamination, because our assay employed dUTP and uracil *N*-glycosylase to limit false-positive results due to carried-over PCR product. The uracil *N*-glycosylase protocol effectively inactivated at least 10^6 copies of deoxyuridine-containing product in intentional-contamination experiments (data not shown). In practice, the specificity of the PCR assay could be improved by performing all assays in duplicate and requiring that the results of the duplicate assays agree before positive results are reported. Although this approach adds considerable expense, it increases confidence in the positive results by providing a level of control for random contamination events.

Twelve patients had no HCMV DNA detected and no positive WBC cultures at any time. Only three (25%) of these patients received ganciclovir therapy, indicating a low index of clinical suspicion of HCMV infection in this patient group. However, one patient in this group developed interstitial infiltrates and had HCMV demonstrated in bronchoalveolar lavage fluid, histologically, on day 34 posttransplantation. The patient was started on ganciclovir on day 42 for presumed HCMV pneumonia, and therapy continued until the patient expired. Both culture and PCR failed to document an antecedent viremia.

Culture of plasma for HCMV is generally considered to be a low-yield procedure and was not done in this study (23). However, plasma may be the best specimen for detection of circulating HCMV DNA. Our data indicate that the yield by PCR from plasma is better than the yield from WBC pellets. This may reflect a larger number of copies of target DNA in plasma or differences in efficiencies and kinetics of amplification of DNA from the two types of specimens. Another potential advantage to using plasma rather than WBC for DNA detection is that patients can be monitored for circulating HCMV during periods of neutropenia. However, almost all of the plasma specimens that contained HCMV DNA were obtained from patients after bone marrow engraftment.

Although few data that allow direct comparison of PCR results for plasma and WBC fractions of blood are available, several groups of investigators have compared the results of plasma PCR with WBC cultures for HCMV. Spector et al. found a good correlation between the two procedures for detection of HCMV in 23 AIDS patients with visceral disease (23). Brytting et al. found a correlation between HCMV DNA in serum specimens and isolation of the virus from WBC for four renal transplant recipients with HCMV disease (3). Wolf and Spector studied the utility of plasma PCR in the early diagnosis of active HCMV infection in 83 BMT recipients and found that PCR was more sensitive than WBC culture for diagnosis of visceral disease (28). Their plasma PCR had a positive predictive value of 60% for disease development and a negative predictive value of 97%.

One potential concern with applying a technology as sensitive as PCR to the detection of a virus that latently infects large number of individuals is that the viral genome would be frequently detected in antibody-positive, asymptomatic individuals. We found no HCMV DNA in plasma or WBC from single blood specimens obtained from 24 healthy, HCMV antibody-positive blood donors (data not shown) and did not consistently find HCMV DNA in specimens from antibody-positive BMT recipients without a high index of suspicion of HCMV disease. In fact, for 32% of the HCMV-seropositive patients, blood specimens collected during the first 100 days after transplantation remained negative for HCMV DNA. Other investigators have used detection of viral mRNA (1) or quantitative PCR methods (7) to facilitate the differentiation between latent and active HCMV infection. We found that the demonstration of HCMV DNA in multiple consecutive plasma samples can also be used to accurately identify the BMT recipients at the greatest risk of developing viremia.

HCMV DNA was demonstrated in the blood samples of five patients after the cessation of preemptive antiviral therapy. No adverse outcome was associated with the persistence of circulating HCMV DNA in these patients. Conversely, viral DNA was not detected in the blood after completion of ganciclovir therapy in the two patients with HCMV pneumonia who had antecedent viremia. Our data suggest that PCR is a poor pre-

dictor of the efficacy of early ganciclovir therapy in BMT recipients. In contrast, Einsele et al. (5) found that persistence of HCMV DNA in blood after cessation of antiviral therapy was associated with an adverse outcome in 6 of 15 BMT recipients with symptomatic infections (40%). Three patients died after initial improvement, and three patients had early relapse of HCMV disease. Einsele et al. found that PCR was a better predictor of the efficacy of antiviral therapy than culture or clinical assessment. It is difficult to compare the results of the two studies, since ours included relatively few patients with HCMV disease and theirs included no patients with asymptomatic HCMV infections.

Plasma PCR is a practical and cost-effective alternative to WBC cultures in our laboratory setting for monitoring BMT recipients. WBC cultures are labor-intensive because of the cumbersome WBC preparation protocol, processing of multiple shell vials, and the inoculation and repeated examination of conventional tube cultures. The HCMV PCR assay can be completed in 2 working days. The current direct cost of reagents, supplies, and labor for an HCMV blood culture in our hospital is $122.25. The direct cost of a plasma PCR assay for HCMV DNA, done in duplicate, is estimated to be $12.13. The average time for detection of positive shell vials in this study was 2.8 days, and that for conventional tube cultures was 13.8 days.

Traditional methods for DNA extraction from peripheral blood WBC and plasma have employed proteinase K digestion followed by phenol-chloroform extraction (4). However, these methods are lengthy, complex, and costly. In addition, they may cause inhibition of DNA amplification because of organic-solvent carryover and are relatively inefficient for recovery of small number of target molecules. We found no difference in target recovery or analytical sensitivity when we compared our simple sample extraction protocol with a traditional proteinase K-phenol-chloroform protocol using a limited number of HCMV-positive WBC and plasma samples. In addition, we found no evidence of inhibition due to heparin in our assay system as judged by experiments in which CMV DNA was added to heparinized plasma and serum specimens obtained from the same individuals.

In conclusion, detection of circulating HCMV DNA in either plasma or WBC is more sensitive than culture for detection of HCMV in the blood of BMT recipients. Onset of viremia was preceded by detection of circulating DNA in all but one of the patients. The predictive value of a positive test for circulating DNA is improved if patients are monitored over time and have multiple consecutive positive samples. We anticipate that the earlier recognition of high-risk patients provided by the detection of circulating DNA may improve the efficacy of preemptive ganciclovir therapy. Future studies will focus on this important issue.

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