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Monitoring of Cytomegalovirus Infection and Disease in Bone Marrow Recipients by Reverse Transcription-PCR and Comparison with PCR and Blood and Urine Cultures

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Preemptive therapy is a promising strategy for the prevention of serious cytomegalovirus (CMV) disease after bone marrow (BM) transplantation but requires relevant diagnostic tests. We compared the clinical value of a reverse transcription (RT)-PCR method, which detected a late viral mRNA in peripheral blood leukocytes (PBL), with a PCR method that detected the viral DNA in PBL and with viral culture from leukocytes and urine for the diagnosis of symptomatic CMV infection after BM transplantation. Forty-five consecutive BM recipients were prospectively tested at weekly intervals by the four methods. CMV infection, demonstrated either by the culture of CMV or by repeated detection of viral DNA, was observed in 28 patients, but only 14 developed CMV-related clinical symptoms. The clinical sensitivity and specificity of each technique for detection of symptomatic infection were, respectively, 36 and 74% for urine culture, 43 and 84% for leukocyte culture, 100 and 65% for PCR, and 71 and 94% for RT-PCR. Although PCR detection of DNA in PBL was the earliest and most sensitive technique for the diagnosis of CMV infection, RT-PCR was more predictive of the onset of CMV-related clinical symptoms. These data suggest that both molecular methods should be used for identifying BM recipients at highest risk of CMV disease.

Cytomegalovirus (CMV) infection is a major cause of mortality and morbidity during bone marrow transplantation (BMT) (24), causing severe complications such as interstitial pneumonia, gastrointestinal disease, and marrow suppression.

Antiviral agents such as ganciclovir and foscarnet, used alone or in combination with intravenous immunoglobulins, have clinical benefits, either preventing (14, 31) or curing (11) CMV disease after BMT. Nevertheless, these therapeutic agents are associated with toxicity and excess cost and must therefore be used to treat only patients at high risk of CMV disease. Thus, rapid and sensitive tests for the diagnosis of CMV infection are important and should ideally be predictive of the occurrence of CMV disease. Serologic tests for specific immunoglobulin G or M antibodies are not reliable as markers of CMV infection in BMT patients because of defective humoral responses and passively transferred antibodies (transfusions or intravenous immunoglobulin administrations). Viral culture from the buffy coat of peripheral blood leukocytes (PBL) has shown a positive predictive value of 60% for the onset of CMV disease during BMT (24) but is rarely positive before 4 to 6 weeks after transplantation, because of granulopenia. Two new methods, the CMV antigenemia assay (6, 30) and PCR detection of CMV DNA in PBL (8–10, 25, 30) or plasma (37) have already proven their usefulness after BMT, showing increased sensitivity for the diagnosis of CMV infection in this setting. However, these techniques are not always relevant to CMV disease status, and the decision to start specific therapy is often difficult.

We have previously developed a reverse transcription-PCR (RT-PCR) method for indirect detection of a late viral mRNA that encodes the major capsid protein of CMV (16). Using this method, we have confirmed the late kinetics of this mRNA and demonstrated a correlation between levels of major capsid protein transcripts and viral replication (16). The clinical value of this method, as a specific marker of CMV disease, has been demonstrated in AIDS patients (17).

The aims of the present study were (i) to investigate the frequency and the clinical consequences of CMV infection after BMT and (ii) to determine the value of major capsid protein mRNA detection as a marker of the onset of CMV disease in this setting, in comparison with viral cultures from PBL or urine or viral DNA detection in PBL.

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MATERIALS AND METHODS

Patients and follow-up. Forty-five consecutive adult patients who received BMT after total body irradiation were enrolled from November 1991 to June 1994. All the patients, 26 males and 19 females, were seropositive for CMV before the transplantation and had a median age of 33 years (range, 18 to 56).

Thirty patients received autologous BMT: 24 with acute myeloblastic leukemias, 3 with acute lymphoblastic leukemias, 2 with stage III multiple myelomas, and 1 with non-Hodgkin's lymphoma. Acute leukemias were autografted while in complete remission, with marrow that had been purged in vitro by mafosfamide (19). The Non-Hodgkin's lymphoma and multiple myeloma patients received CD34 purified marrow (15).

Fifteen patients received allogeneic BMT: 7 with acute myeloblastic leukemias, 1 with acute lymphoblastic leukemias, 2 with multiple myelomas, 3 with Philadelphia positive chronic myeloid leukemia, 1 with severe aplastic anemia, and one with acute lymphoblastic leukemia transformation of primary myelofibrosis.

All patients received as prophylaxis of infection the following: polyvalent intravenous immunoglobulins (Biotransfusion, Les Ulis, France) at 250 mg/kg of body weight weekly until discharge, acyclovir at conventional doses for herpes simplex virus infection (250 mg/m^2 intravenously three times daily), and total gut decontamination. Prophylaxis for graft-versus-host disease consisted of cyclosporine A and methothrexate.

For symptomatic CMV infections, or for allogeneic recipients with a positive viremia, ganciclovir (5 mg/kg twice a day for 14 days) was given in combination with specific CMV hyperimmune globulins (Biotransfusion) (11) (400 mg kg/day

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for 4 days and then, 250 mg/kg weekly for 2 months). In most cases, maintenance therapy (ganciclovir, 5 mg/kg daily) was given for 2 months.

Throughout hospitalization and, in most cases, posthospitalization, all patients were monitored weekly for CMV infection. A median of 11 specimens (range, 5 to 28) per patient were tested by the four methods. The median duration of monitoring was 105 days (range, 35 to 330).

Leukocyte and urine cultures. Leukocytes and urine were cultured by using a detection of early antigen foci, as previously described (1). Briefly, the buffy coat of 10 ml of heparinized blood, or 500 μl of urine, was inoculated into 24-well plates containing a confluent monolayer of human embryonic fibroblasts. Plaques were centrifuged (2,000 × *g* for 45 min), and the immediate-early proteins ppUL122 and -123 were detected 48 h later, with a monoclonal antibody (clone E13; Argène, Varhiles, France), in a classical immunoperoxidase reaction (1).

Detection of viral nucleic acids in PBL. Total leukocytes from 10 ml of heparinized blood were isolated on Histopaque 1119 (Sigma, L'Isle d'Abeau Chesnes, France) and stored at −80°C (10^5 to 10^6 cells per tube). PCR and RT-PCR were carried out as previously described (16, 17), with minor modifications: DNA was extracted by cell lysis in the presence of proteinase K (100 μg/ml), and PCR was directly performed on the cell lysis supernatant. Total RNA was extracted with the RNAzol RNA extraction system (Bioprobe, Paris, France) and dissolved in diethyl pyrocarbonate-treated water. Half of the total RNA extraction was reverse transcribed in the presence of 100 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, Md.), 20 U of RNAsin (Boehringer, Mannheim, Germany), 50 pM random hexanucleotides (pdN6; Pharmacia), and 0.2 mM each deoxynucleoside triphosphate (dNTP). The cDNA was then amplified in the same mix as genomic DNA. The PCR mix consisted of 50 pM primers CMCP1 and CMCP2 (12), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM each dNTP, and 1.25 U of cloned *Taq* polymerase (Perkin-Elmer Cetus, Emeryville, Calif.) added to the DNA template in a total volume of 100 μl. Forty cycles of amplification (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 s) were done in a thermal cycler. Aliquots (25 μl) of each amplification product were electrophoretically separated in a 2% agarose gel, transferred in alkaline conditions to nylon filters (Hybond N+; Amersham, Les Ulis, France), and hybridized with the ^{32}P-kinase-labeled oligoprobe CMCP 3 (16).

Controls. All the recommended precautions (18) to avoid contamination by PCR product carryover were taken, including physical separation of the pre- and post-PCR steps and use of filtered tips. Two negative and positive controls were included in each experiment.

To determine whether cell specimens were suitable for DNA amplification, all the negative samples were controlled by amplification with primers flanking the β-globin gene as described elsewhere (28). To confirm that RNA samples were free of contaminating DNA, PCR was performed on the remaining RNA sample without the reverse transcription step.

Definitions. CMV infection was defined by positive isolation of CMV from any site or DNA detection in two consecutive blood samples. The requirement for consecutive PCR-positive blood samples minimized the inclusion of patients with sporadic DNA detection in their blood (25), either due to DNA contamination of the PCR assay or true sporadic DNAemia. Several episodes of CMV infection can occur in one patient; CMV infections separated by three consecutive specimens that tested negative by all the techniques were defined as different episodes of CMV infection.

Symptomatic CMV infections were defined as CMV infections that were associated with either (i) a proven visceral disease (20), e.g., involvement of the lungs or gastrointestinal tract, as proven by CMV detection in bronchoalveolar lavage or gastrointestinal biopsy, and compatible clinical manifestations, or (ii) a presumptive viral syndrome (20), e.g., unexplained fever for at least 72 h associated with either late (>30 days) leukocyte recovery (defined by two consecutive leucocyte counts of over 1,000/μl) or secondary leukopenia (defined by a progressive decline of the leukocyte count to below 500/μl occurring after leucocyte recovery).

Statistical analysis. The clinical performances of the four different tests in the detection of CMV symptomatic infection were assessed by the sensitivity, specificity, and positive and negative predictive values defined, respectively, by the proportion of symptomatic patients with positive test results, the proportion of asymptomatic patients with negative test results, the proportion of patients with positive test results who developed symptoms, and the proportion of patients with negative test results who remained asymptomatic (2, 3). These values were compared by Fisher's exact test, as were all the qualitative variables.

Times to the leukocyte engraftment were compared between different groups of patients by the median test.

The median time points of onset of positivity of the tests were compared by log-rank analysis.

RESULTS

CMV infection. To identify patients with CMV infection, 576 blood and urine specimens from the 45 patients were collected weekly and tested by culture and PCR.

With these methods, 28 patients (62%) were found to be infected during the monitoring, developing 37 episodes of CMV infection. The median onset of CMV infection was 21 days posttransplantation (range, 0 to 75 days). No difference in the prevalence of CMV infection was observed between the recipients of allogeneic and autologous BM.

TABLE 1. Positivity of the four methods with CMV infections

Test	No. of infected patients (n = 28)	No. of CMV infection episodes (n = 37)	Median day of onset[a] (range)
PBL culture	11	13	42 (24–70)
Urine culture	14	19	56 (0–75)
PCR	25	33	24 (0–63)
RT-PCR	12	15	30 (0–71)

[a] Day 0 is the day of transplantation. The data reflect only the first CMV episode for each patient.

The results of each test for the 28 CMV-infected patients are summarized in Table 1. PCR detection of CMV DNA was the most sensitive method, identifying 25 infected patients and 33 episodes of CMV infection. The sensitivity of viral culture from blood and urine specimens, for the detection of CMV infection, was poor (respectively, 39 and 50%). PCR was also the earlier method (median time to positivity, 24 days) compared with positivity of viral cultures from blood and urine (respectively, 42 and 52 days; $P < 0.0001$ [log-rank test]).

Late-mRNA detection was positive in 12 of the 28 infected patients. No mRNA was detected without another marker of CMV infection, except for one autologous BM recipient who had two blood samples positive for mRNA (at 35 and 42 days posttransplantation), whereas the other three tests remained negative; the specificity of mRNA detection for the detection of CMV infection was thus 94%.

CMV-related symptoms. Fourteen patients (31%) including five allogeneic and nine autologous BM recipients had symptomatic infections. The median time to onset of symptoms was 42 days posttransplantation (range, 14 to 73 days). Symptomatic infections included proven visceral disease (six episodes of pneumonia in five patients and colitis in one patient) or presumptive viral syndrome (n = 8). The viral syndrome always combined fever with altered hematopoiesis, which led to a delay in leukocyte engraftment (n = 4) or secondary leukopenia (n = 5). In three patients, cytopenia was associated with liver biochemistry abnormalities, but no liver biopsies was performed. Visceral sites of CMV disease were also often associated with altered hematopoiesis (in the patient with CMV colitis and three of the five patients with CMV pneumonia). As a result, the median time to leukocyte engraftment was significantly longer in patients with symptomatic CMV infection (median, 32.5 days) than in patients free of CMV infection (median, 22 days; $P = 0.05$ [median test]).

The CMV pneumonia was more frequent in allogeneic than in autologous BMT recipients (4 of 15 versus 1 of 30; $P = 0.04$ [Fischer's exact test]). However, if we consider the prevalence of all CMV symptoms, no difference was observed between the allogeneic and autologous BMT recipients.

Clinical relevance of the four diagnostic tests. The clinical values of each test are summarized in Table 2.

Positive leukocyte culture had a positive predictive value (PPV) of 55% for the onset of symptomatic CMV infection but showed poor clinical sensitivity and moderate negative predictive value. The culture of CMV from urine had less clinical relevance, because of lower sensitivity and specificity.

The PCR method was positive for all 14 symptomatic patients. In 13 patients, DNA detection preceded, or was simultaneous with the onset of symptoms; the median overall inter-

TABLE 2. Clinical relevance of assays for the detection of CMV symptomatic infections

Test	%			
	Sensitivity	Specificity	PPV	NPV[a]
PBL culture	43 (6/14)	84 (26/31)	55 (6/11)	76 (26/34)
Urine culture	36 (5/14)	74 (23/31)	38 (5/13)	72 (23/32)
PCR	100 (14/14)	65 (20/31)	56 (14/25)	100 (20/20)
RT-PCR	71 (10/14)	94 (29/31)	83 (10/12)	88 (29/33)

[a] NPV, negative predictive value.

val between DNA detection and disease onset was 11.5 days. However, the clinical specificity of PCR was only 65%, since 11 patients had repeatedly positive DNA detection without developing any symptoms.

The RT-PCR method had better clinical specificity (94%; $P = 0.01$ [Fisher's two-tailed exact test]) and the best PPV (83%) of all the evaluated techniques. However, its clinical sensitivity was only 71%. Nevertheless, for the 10 patients with symptomatic CMV infection and late mRNA detected, the test was always positive before, or at, the onset of symptoms; the median interval between RNA detection and CMV symptoms onset was 4 days.

DISCUSSION

The development of CMV infection in 62% of the patients in this study confirms the importance of CMV infection during BMT and emphasizes the need for improved methods of identifying CMV infection. The use of a sensitive PCR-based technique appeared very useful for the study population, especially during the first month after transplantation, when the leukocyte count is low. The sensitivity of leukocyte culture was indeed very poor during that period, supporting this notion. The culture technique used in this study involved detection of an immediate-early antigen of CMV, 48 h after inoculation. Culture sensitivity would probably have been improved had immunostaining also been performed at a later time point and if standard cultures were associated. Nevertheless, rapid commencement of specific therapy when the infection, demonstrated by DNA detection, became symptomatic, could also account for the poor sensitivity, especially when treatment was started before full leukocyte recovery.

Clinical symptoms of CMV infection were present in only 14 of the 28 patients who had one or more episodes of CMV infection. This means that more than half of CMV infections remained totally asymptomatic. Preemptive therapy is a promising strategy for the management of CMV infection in BM recipients (8, 14, 31), and a test which could predict symptomatic infection would be of great value. Because of their poor PPV, neither blood or urine culture nor DNA detection in PBL by PCR seems appropriate for this purpose. In contrast, detection of viral late mRNA by the RT-PCR method has a high PPV, suggesting the potential usefulness of this new marker to indicate patients at highest risk of symptomatic infection. The good PPV was due to its very high clinical specificity, in keeping with a study involving AIDS patients in which this marker was also very specific for CMV disease (17). However, this method failed to detect all the symptomatic episodes (clinical sensitivity, 71%) and therefore should be performed in combination with a more sensitive technique for the monitoring of BMT recipients. In this regard, viral DNA detection by PCR appears very useful given its 100% negative predictive value for CMV disease.

In a recent study involving renal transplant recipients (23), the clinical value of late-mRNA CMV detection was very similar to that obtained with the pp65 antigenemia test. As the latter is cheaper and less time-consuming than RT-PCR, we are now investigating whether it also performs well in BMT recipients. However, a previous study using the pp65 antigenemia technique in allogeneic BMT (6) found a clinical PPV of only 53% in recipients not receiving prophylactic ganciclovir. In liver transplant recipients, the RT-PCR method had very good clinical specificity for symptomatic CMV infection but a sensitivity of only 25% (26). However, the samples used in the latter study were mononuclear cells, not total PBL, and, because polymorphonuclear cells are a major target population to be infected during active CMV infection in immunocompromised hosts (7, 13, 29), this could account for the low sensitivity.

The effects of CMV infection on BM engraftment have previously been reported mainly in terms of platelet recovery (34–36). Because 24 of our 45 patients were autografted with mafosfamide-purged marrow, which delays platelet engraftment (19), we did not include this as an indicator of CMV disease. Furthermore, in allogeneic BM recipients, acute graft-versus-host disease can cause prolonged isolated thrombocytopenia (12). However, we noted a slight, albeit significant delay in leukocyte recovery in patients with CMV symptomatic infection. This supports in vitro studies showing the impact of CMV on hematopoiesis, involving either direct infection of hematopoietic progenitors (21, 32, 33) or indirect impairment of hematopoiesis through infection of either stromal (4, 32) or accessory (22) cells. At last this finding may provide further support for starting anti-CMV therapy when patients develop CMV infection associated with hematologic disorders, even with a myelotoxic drug such as ganciclovir. Recent reports of hematologic improvement or successful engraftment on ganciclovir therapy prescribed for a symptomatic CMV infection (5, 27) support this view.

In conclusion, CMV infection remains a major problem following BMT, because of its high prevalence and clinical consequences, particularly for marrow function. Accurate and clinically relevant virological markers are still needed, for prompt diagnosis. The value of sensitive PCR-based methodology, for identification of CMV-infected recipients who are candidates for preemptive therapy (8, 10, 25) or for monitoring the ganciclovir treatment (9), has already been demonstrated. Combination of PCR with an RT-PCR technique detecting a late viral mRNA allows the optimization of the monitoring of marrow recipients and may provide some new insights into the pathogenesis of CMV infection and disease in immunosuppressed patients. Both of these techniques remain to be compared with the pp65 antigenemia test but can already be used for clinical trials comparing different preemptive therapeutic strategies for bone marrow recipients.

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