Clinical Trial of Quantitative Real-Time Polymerase Chain Reaction for Detection of Cytomegalovirus in Peripheral Blood of Allogeneic Hematopoietic Stem-Cell Transplant Recipients

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The preemptive therapy of cytomegalovirus (CMV) reactivation is useful for the prevention of CMV disease in allogeneic hematopoietic stem-cell transplant (HSCT) recipients. We compared results of the pp65 CMV antigenemia test with quantitative touch-down polymerase chain reaction (Q-PCR) on unfractionated whole blood for the detection of CMV reactivation in 51 HSCT recipients. Forty episodes of reactivation in 28 patients were detected by antigenemia and treated by antiviral drugs. Q-PCR detected CMV DNA in 39 (97.5%) of 40 reactivation episodes. False-positive results occurred in 3% of tests, of which 63% were borderline positive. Q-PCR results were positive earlier than antigenemia results in 30 (77%) of 39 episodes detected by antigenemia. Q-PCR remained positive after treatment was discontinued in 14 (36%) of 39 episodes and predicted the return of CMV reactivation in 4 (31%) of 13 episodes. Q-PCR was more sensitive than the antigenemia test and had sufficient specificity for clinical use.

The administration of antiviral therapy for cytomegalovirus (CMV) on the basis of evidence of reactivation has become a common strategy in the treatment of hematopoietic stem cell transplant (HSCT) recipients [1]. A commonly used test to detect CMV reactivation is the immunoperoxidase staining of CMV lower-matrix protein pp65 (UL83) in peripheral blood neutrophils [2]. This test for CMV pp65 antigen, as done at the National Institutes of Health (NIH), requires the microscopic examination of 400,000 neutrophils from peripheral blood (antigenemia). In our allogeneic HSCT recipients, a single neutrophil with pp65 is considered to be sufficient to begin therapy with ganciclovir, valganciclovir, or foscarnet, all of which are expensive and potentially toxic drugs. Therapy is continued until the results of the antigenemia test, done weekly, are negative for 3 consecutive weeks. Although it is unknown whether this strategy leads to excessive antiviral therapy, it is clear that the incidence of CMV disease is now very low in our patients. The limitations of the antigen test in detecting reactivation are the cost of technician time, need for technical expertise, inability to test stored blood, and requirement of neutrophil counts $>0.5 \times 10^9$ cells/L. Furthermore, in some patients, there is a failure to detect reactivation before the onset of clinical disease. The present study was done to evaluate the ability of an in-house quantitative polymerase chain reaction (PCR) to replace the antigen test.

PATIENTS, MATERIALS, AND METHODS

Patients

Fifty-one patients aged 8–65 years were entered into the study between 4 January 2000 and 12 February 2002.
All were enrolled prior to transplantation and subsequently received HSCT from HLA-matched, related donors. Twenty patients received T-cell–depleted peripheral blood stem cells (PBSCs) as part of a myeloablative regimen. Thirty-one patients received a T-cell–replete PBSC transplant after nonmyeloablative conditioning. Patients were selected for enrollment if either the recipient (R+) or the donor (D+) had IgG antibody to CMV, as tested by ELISA (Zeus Scientific). Thirty-four patients were D+/R+, 10 were D+/R−, and 7 were D−/R+. Patients who were D+/R− were excluded from the study. Indications for HSCT were hematologic malignancy in 29 patients, renal cell carcinoma in 14 patients, nonmalignant hematologic disease in 5 patients, and other solid-organ tumors in 3 patients. Shell-vial culture for CMV was not used to determine preemptive therapy. Patients were tested by the antigen test and Q-PCR 24 h prior to, the day of, or 24 h after receiving the stem cells and weekly up to day 100 after transplant, or later, if clinical suspicion arose. If the leukocyte count was less than $\sim$1000 cells/μL, Q-PCR, but not antigen, was tested. A clinician not blinded to the Q-PCR results collected clinical data prospectively on all patients. In this NIH-approved protocol, clinicians caring for the patients were not informed of the results, so that preemptive treatment was not based on Q-PCR results. Preemptive therapy with ganciclovir (5 mg/kg every 12 h or foscarnet 120–180 mg/kg per day in 2–3 divided doses) was initiated when at least 1 antigen-positive cell was found in 400,000 leukocytes and was continued until the results of 3 consecutive tests were negative. Drugs were changed if antigenemia continued for 3 weeks or if antigenemia recurred within 2 weeks of discontinuing therapy. Follow-up of surviving patients ended on 2 July 2002. Informed consent was obtained from all study participants or their parents or guardians. The clinical research was approved as protocol 00-I-0059 by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases of the NIH and was in compliance with the guidelines of the US Department of Health and Human Services.

Definitions
“Treatment” indicates the use of at least 7 days of ganciclovir, valganciclovir, or foscarnet. Prophylactic acyclovir (800 mg orally, 4 times/day or 500 mg/m² intravenously every 8 h) was given to all patients in the study from day −7 to +100 after transplant and was not included in the definition of treatment. An “episode” of CMV reactivation was defined as treatment based on either the presence of CMV antigen in neutrophils or the clinical suspicion of disease. “Relapse,” or “reactivation,” was defined as an episode that occurred >14 days after a prior reactivation. A “false-positive” Q-PCR result was defined as a positive Q-PCR test in the absence of any other evidence of CMV reactivation. This required no positive antigen test and no other positive Q-PCR result within 8 days of the false-positive results and (to insure that treatment did not mask clinical CMV disease) no treatment within 14 days before or after the positive Q-PCR result.

Quantitative Touch-Down Real-Time PCR (Q-PCR)

Nucleic acid extraction. Blood samples were collected in 5-mL EDTA-treated tubes for the Q-PCR assay. After thorough mixing, a 200-μL aliquot of whole blood was transferred to a microcentrifuge tube that contained 0.9 mL of the lysis buffer included in the NucliSens isolation kit (bioMérieux). The microcentrifuge tube was vortexed, coded with a unique identifier to blind the sample, and stored at −70°C until extraction. The NucliSens isolation kit (bioMérieux) was used according to the manufacturer’s instructions to extract total nucleic acid from the whole-blood aliquot. In brief, cells in the sample were lysed, and silica particles were added, to bind with the nucleic acid. After several washes, the nucleic acid was eluted from the silica using 50 μL of elution buffer. Positive and negative controls were included with each batch of patient specimens extracted.

The positive control consisted of a 200-μL aliquot of normal donor EDTA-treated blood spiked with whole CMV (ATCC VR-807), and the negative control consisted of a 200-μL aliquot of normal donor EDTA-treated blood that had been determined to be CMV negative using the Q-PCR assay.

Q-PCR internal control (IC). To verify that impurities that may contribute to Q-PCR inhibition were completely removed during the extraction procedure, an IC that was amplifiable by the CMV primers was constructed [3]. The concentration (number of copies per microliter) was calculated using the absorbance at 260 nm optical density value and the plasmid molecular weight. The plasmid was linearized and then diluted in TRIS-EDTA (TE) buffer (pH 8.0) with glycerol (33.3 μg/mL), to obtain the working stock concentration, and then stored at −70°C.

Q-PCR standards. A plasmid that contained the amplification site was generated by cloning the target region, located on the CMV glycoprotein-B gene, into the pCR2.1 vector (Invitrogen). After propagation and purification of the plasmid, the concentration was calculated as described above. The plasmid was linearized and then diluted in TE buffer (pH 8.0) with glycerol (33.3 μg/mL), to obtain concentrations of 5000, 500, 50, and 5 copies/reaction. Aliquots of the standards were prepared in single-use tubes and stored frozen at −70°C.

DNA amplification. Q-PCR [4] was done using the LightCycler (Roche Molecular Biochemicals). This instrument provides a platform for the amplification and continuous monitoring of the accumulation of target amplicons through the application of fluorescence resonance energy transfer (FRET) technology using 2 fluorophore-labeled hybridization probes [5]. PCR primers CMV-1.up (5′-GCC GAC GGG ACC ACC GTG ACG-3′) and CMV-2.down (5′-GCT CGC TGC TCT GCG

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TCC AGA C-3′ (Idaho Technologies) were used to amplify a 203-bp region of the CMV glycoprotein-B gene. All reactions were done in glass capillaries (Roche) using 1× LightCycler-FastStart DNA Master Hybridization Probes reaction mixture (Roche), which contained FastStart Taq, reaction buffer, dNTP, and final concentrations of 1.0 μmol/L for each primer, 0.2 μmol/L for each CMV FRET probe, 4.0 μmol/L MgCl₂, and 1 U of heat-labile uracil-DNA glycosylase (UNG) (Roche). In the reaction mixture, dUTP was substituted for dTTP. In reactions used to evaluate the inhibition of amplification, 500 copies of the IC and 0.2 μmol/L of each mimic FRET probe were also added. Each reaction tube contained 15 μL of master mix and a 5-μL aliquot of the extracted DNA or sterile water. FRET probe sets for both the CMV target and the IC were designed to anneal with a 1-base gap between the probes and were synthesized by Idaho Technologies. For CMV detection, the following probe set was used, with the reporter probe being labeled with Red 640: CMV-FRET.1 (5′-GTG TTT ATA ATT CTG GTC GCA AAG GAC-fluorescein-3′) and CMV-FRET.2 (5′-Red 640-GGG ACC ACC GTC GTC TGA TGC A-3′). For IC detection, the following probe set, labeled with Red 705 as the reporter, was used: Mimic-FRET.1 (5′-GAT ATC GTG CAT TCC GAC AGC ATC-fluorescein-3′) and Mimic-FRET.2 (5′-Red 705-CCA GTC ACT ATG GCG TGC TGC TAG-3′). The thermocycling protocol consisted of an initial 10-min incubation at 30°C for UNG activity to take place, followed by an 8-min incubation at 95°C to activate the DNA polymerase, inactivate UNG, and melt double-stranded DNA. Then a touch-down procedure followed that consisted of 5 s at 95°C, annealing for 10 s at temperatures decreasing from 66°C to 54°C during the first 12 cycles (with 1°C decreasing steps for each cycle), and an extension step at 72°C for 20 s. The annealing temperature for the remaining 34 cycles was 54°C for 10 s. Touch-down PCR, with decreasing annealing temperatures, was used because it has been shown to have increased sensitivity to low copy numbers in some applications [4]. In each experiment, duplicates of the standards (5000, 500, 50, and 5 copies/reaction) were included to generate a standard curve for the quantification of positive patient samples, and each sample was tested in 3 reactions. One reaction contained the IC and was not used for quantification purposes because the concurrent IC amplification might have interfered with the accuracy of the quantification. A negative CMV result had to have a positive result for the IC to be considered valid, to ensure the absence of inhibitors in the specimen. All acquired fluorescence data were analyzed using LightCycler software. The conversion of CMV DNA values obtained by Q-PCR to copies per milliliter was done as follows. DNA extracted from 200 μL of whole blood was suspended in 50 μL of water, of which 5 μL was used in each of 2 reactions. The sum of the 2 reactions represented 40 μL of whole blood, for which reason the sum was multiplied by 25 to obtain copies per milliliter.

**CMV Antigenemia**

Blood samples were collected in 10-mL EDTA-treated tubes and processed the same day for the antigenemia assay. A 5-mL aliquot of whole blood was added to 30 mL of ammonium chloride potassium bicarbonate lysing buffer (Biofluids) and agitated for 5 min, to lyse the red blood cells. The blood mixture was then centrifuged at 500 g for 5 min, and the supernatant was discarded. After washing the pellet once with 3% bovine serum albumin (BSA)-PBS, the leukocytes (white blood cells; WBCs) were resuspended in 3% BSA-PBS to a final concentration of 2.0 × 10⁶ WBCs/mL. Two slides per specimen were prepared by spotting 0.1 mL of the cell suspension (2 × 10⁵ WBCs) to each slide using a cytocentrifuge (100 g for 3 min). The slides were air dried and then fixed and stained using the CINA kit for CMV antigenemia (Argene), according to the manufacturer’s instructions. Both slides were examined, and the presence of ≥1 antigen-positive cell/4 × 10⁵ WBCs was considered to be a positive result.

**RESULTS**

**Comparison of Plasma and Whole-Blood Q-PCR**

In the first 91 samples, both plasma and whole-blood assays were done on each sample. Of 53 samples that were positive by Q-PCR in whole blood, only 36 (68%) were positive in plasma. In 38 samples negative in whole blood, only 5 (13%) plasma samples were positive, of which 4 were borderline (as defined below). Because plasma appeared to have a lower CMV copy number than whole blood, further testing of plasma was not done. Similar results have been reported by others [6, 7].

**Test Reproducibility at Low DNA Copy Numbers**

The repeatability of values ≤5 copies/reaction, which was the bottom standard, was assessed by retesting the DNA extracted from 65 whole-blood specimens in which 1 of the paired reaction tubes gave copy numbers ≤5. If both of the prior reactions were positive, repeat testing of paired samples gave a positive in at least 1 tube in 20 (87%) of 23. If 1 of the pairs was negative in the first run and the other sample in the pair had ≤5 copies, then repeat testing found at least 1 tube to be positive in only 21 (50%) of 42 samples. This result was interpreted to mean that the repeatability of a positive test was acceptable at low copy numbers unless 1 of the paired samples was negative and the other reaction was positive at ≤5 copies. In subsequent analyses, results with 1 tube positive at ≤5 copies and the other tube negative were designated as borderline positives. This designation indicated that the test might not be repeatable as a positive. Unless qualified as “borderline,” the
Figure 1. Two examples of a typical positive quantitative touch-down real-time polymerase chain reaction (Q-PCR) tests prior to a positive antigenemia result. Solid bars, treatment for cytomegalovirus (CMV) reactivation. CMV DNA was detected 2 weeks before the detection of antigenemia. Ag, antigen.

Figure 2. Quantitative touch-down real-time polymerase chain reaction (Q-PCR) and antigen (Ag) test results in a patient with cytomegalovirus (CMV) colitis and severe graft-versus-host disease (GVHD). Gastrointestinal bleeding was present on transplant day 29, when results of both tests were negative. Treatment for CMV was begun 1 week later, when results of both tests were positive (solid bar). Arrow, The day that colonic biopsy testing revealed CMV disease. Cross, Time of death from Candida sepsis: both GVHD and CMV disease were noted in the colon at autopsy.

Comparison of Q-PCR and Antigenemia Results

CMV episodes evaluated. A total of 966 samples were assayed by Q-PCR. For 831 samples, an antigen assay was done on a sample collected within 3 days of the Q-PCR; the exceptions occurred when neutropenia in a patient prevented the determination of antigen testing. Of the 51 patients, 28 (55%) had a total of 40 episodes of CMV reactivation. Three additional patients were treated but were not considered to have had an episode of reactivation. Two patients were treated because of clinical suspicion but did not have positive Q-PCR results, a positive antigen test, or confirmed CMV disease. A third patient was treated because of clinical suspicion and never had a positive antigen test but did have positive Q-PCR test results 8 and 22 days before treatment. This patient had no other evidence of CMV reactivation.

Sensitivity. Q-PCR detected reactivation in 39 (97.5%) of 40 episodes. The exception was a patient who was treated because of a positive antigen test result but who had a negative Q-PCR result. This patient had a positive antigen test only on a single occasion, with a single positive neutrophil among 400,000 neutrophils evaluated.

Peak values. The quantitation of CMV DNA detected by PCR was not found to offer additional information beyond determining that the value exceeded the threshold. Peak values obtained early during an episode did not predict the number of days of treatment before the PCR result became negative. For our analysis, 16 peak values (range, 175–1700 copies/mL), obtained from episodes in which >1 PCR result was positive, did not correlate with the duration of PCR positivity (7–133 days), nor did the peak DNA value correlate with peak antigenemia values or with relapse.

Earlier detection of reactivation. A positive Q-PCR result often preceded a positive antigen result, although the copy numbers were low, as shown in figure 1. Tallying episodes of at least 1 Q-PCR positive result within 8 days prior to therapy, accompanied or not by prior or subsequent positive Q-PCR results, Q-PCR positivity preceded a positive antigen test in 30 (77%) of 39 episodes. In these episodes, the Q-PCR showed a positive result a mean of 14 days before the antigen test. In 5 episodes, the Q-PCR result was first positive on the same day as the first antigen test. In 4 episodes, the Q-PCR test was positive later than the antigenemia test by 7–42 days. If borderline results were excluded, Q-PCR positivity preceded antigenemia in 22 (56%) of 39 episodes by an average of 13.5 days.

Detection of clinical CMV disease. Only 1 patient was...
diagnosed as having CMV disease while participating in the study. This patient developed life-threatening gastrointestinal bleeding during severe graft-versus-host disease (GVHD) on day 29 after transplant. Both the antigen test and Q-PCR were negative at the onset of symptoms. One week later, antigenemia was observed (8 cells), and ganciclovir was administered. Colonic biopsy done 4 days later showed GVHD as well as CMV colitis, which was confirmed by immunoperoxidase staining. He died of *Candida lusitaniae* sepsis 3 weeks after starting antiviral therapy (figure 2).

**Positivity persisting beyond treatment.** Treatment was usually continued until 3 consecutive antigen tests were negative, but the Q-PCR results, which were not reported to the physician, often remained positive. Specifically, Q-PCR remained positive in 29 specimens after therapy in 13 episodes in 12 patients (figure 3). Values were borderline, as defined above, for 11 of 29 specimens. Persisting PCR positivity was followed by relapse (antigenemia recurrence and treatment given) in only 4 of these 13 episodes. Figure 4 shows the Q-PCR value and the number of days after treatment. Of the 4 patients who had a relapse, the peak posttreatment Q-PCR values were borderline in 2 patients and were 1125 and 3100 copies/mL in the other 2. As shown, prolonged low-level PCR positivity was observed that was not necessarily a prelude to relapse. Three of these patients have been followed for at least a year with no evidence of relapse. It is likely that cellular immunity was sufficient to prevent emergence of CMV disease.

**False-positive tests.** Patients who had at least 1 episode of reactivation also had occasional false-positive results, as defined above, which occurred in 17 samples from 13 patients outside an episode. Of the 17, 12 had borderline values. The same DNA extracts were retested on 10 specimens and were negative in 8.

A less ambiguous estimate of the frequency of false-positive results is the frequency of positive Q-PCR results in patients who did not experience a reactivation episode. There were 13 positive Q-PCR in 367 samples (3.5%) from 5 of 22 such patients. Seven of 13 positive results were in the borderline range. Of note, these 22 patients included all 10 D+/R−/H1002 patients enrolled in the study, none of whom had a reactivation episode. Considering patients both with and without reactivation episodes as sources of false positivity, false-positive results occurred in 30 (3.1%) of 966 of tests done and in 18 of (35%) 51 of all patients studied. The number of false-positive Q-PCR tests was decreased to 11 (1.1%) when borderline positive results were excluded. With this more restrictive definition of positivity, no additional episodes of reactivation were undetected. In sum, false-positive results were uncommon, and, in two-thirds of the specimens, the values were in the borderline range. Although it would be useful to compare false-positive PCR test results with those obtained with the antigen test, the calculation is meaningless because, in our patients, all positive antigen tests resulted in treatment for at least 7 days. Therefore, all positive antigen test results met our criteria for an episode of reactivation and would be considered to be “true” positives.

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**Figure 3.** Two examples of quantitative touch-down real-time polymerase chain reaction (Q-PCR) positivity after treatment ended. A, The results of Q-PCR were borderline positive at the end of a course of therapy (arrow pointing left). The amount of cytomegalovirus (CMV) DNA increased in the absence of clinical disease. Treatment was resumed when a single positive cell was found on pp65 antigen (Ag) testing. B, The results of Q-PCR increased progressively after therapy was discontinued, but the patient had no evidence of CMV disease at day 145 after transplant or at a clinic visit 3 months later.

**Figure 4.** Quantitative touch-down real-time polymerase chain reaction (Q-PCR) values obtained after therapy from 12 patients. Solid circles. Results from 4 patients who had reactivation according to the results of a pp65 antigen test and were retreated. Eight patients were not treated again for cytomegalovirus (CMV) and did not develop CMV disease.
DISCUSSION

CMV disease causes severe morbidity and mortality in HSCT recipients, and the risk varies with the patient’s prior exposure to CMV, the source of stem cells, the transplantation regimen, and the presence of GVHD. Intravenous ganciclovir prophylaxis begun at engraftment has been successful in delaying the onset of CMV disease, but neutropenia has remained a serious side effect [8]. Also, the required duration of prophylaxis has not been clearly defined. Preemptive therapy based on early evidence of reactivation has been a popular strategy [1, 2, 8]. The detection of reactivation using the antigen test has proved to be more useful than shell-vial culture [9]. It remains unclear how often patients with asymptomatic CMV reactivation, based on antigenemia, would progress to CMV disease if they were not treated. Both the cost and toxicity of preemptive ganciclovir, valganciclovir, and foscarnet are of concern. However, the low incidence of CMV disease during antigenemia monitoring has supported the use of preemptive therapy based on antigenemia. Ganciclovir resistance, which is manifested as the failure of antigenemia to decrease with treatment, occurs particularly in patients who are receiving intensive immunosuppression for severe GVHD. Changing the regimen to foscarnet usually leads to the clearance of antigenemia. Insensitivity of the antigen test (i.e., progression to CMV disease despite repeatedly negative p65 test results) is uncommon in our patient population but has occurred and usually manifests as gastrointestinal CMV disease (figure 2).

Several centers have reported the advantages of using real-time PCR to evaluate the CMV load in HSCT and solid-organ transplant recipients [10–12]. These advantages include the ability to test blood during neutropenia, to analyze mailed-in samples hemolyzed during shipment, and to detect CMV disease missed by antigenemia. Periods of neutropenia were often short in our patients, and no episodes of CMV disease were recognized during neutropenia. Too few hemolyzed specimens were tested by Q-PCR in our study to evaluate this advantage. We could not evaluate the increased sensitivity to predict CMV disease, because only 1 patient developed CMV disease during the study period. The Q-PCR did detect reactivation an average of 13 days earlier than the antigen test, which is an advantage that has also been reported for a real-time PCR assay of similar sensitivity (i.e., a lower limit of detection of 20 copies CMV DNA/mL vs. 25 copies for our assay) [10]. The delay in treatment did not result in episodes of clinical CMV disease during our study. The Q-PCR also detected persisting CMV DNA after the treatment of 13 episodes had ended. Although virus load at the end of therapy has predicted relapse in solid-organ transplant recipients [11], only 4 of 13 episodes we studied were followed by relapse, and the peak virus load did not predict relapse (figure 4). Virus load at the onset or peak of CMV activation has been correlated with the appearance of CMV disease in a patient population of both HSCT and solid-organ transplant recipients [12], but our patients had too few episodes of disease to confirm this correlation. Despite the high specificity and sensitivity of the Q-PCR assay for the detection of CMV reactivation, none of the intended advantages over the antigen test were detected in our patients. The results cannot be generalized beyond our patient population, considering the diversity of patient populations and treatment regimens currently in use in transplant centers.

References