Diagnosis and Monitoring of Human Cytomegalovirus Infection in Bone Marrow Transplant Recipients by Quantitative Competitive PCR

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Objectives: **Human cytomegalovirus (HCMV) is a common cause of infection worldwide. Severe cytomegalovirus disease is usually observed in immunodeficient individuals such as bone marrow transplant (BMT) or AIDS patients. In these patients, proof of viral presence is not enough for making clinical decisions; one must report the quantity of virus or viral load in appropriate clinical specimens to demonstrate the relationship between disease severity and HCMV infection. The goal of this study was to use quantitative competitive polymerase chain reaction (PCR) to determine HCMV viral load in 26 BMT recipients.**

Materials and Methods: **Peripheral blood was collected weekly for 100 days from 26 BMT recipients. Qualitative and quantitative competitive PCRs on 10^5 mononuclear cells were performed for each patient. The same tests were performed once for each of 26 donors. In addition, the anti-HCMV humoral response was detected by performing IgM and IgG ELISAs in donors and recipients prior to transplantation.**

Results: **Of 26 BMT donors and recipients, 25 and 26 were IgG positive, and 2 and 6 had HCMV-specific IgM antibodies, respectively. From 313 total clinical specimens tested, 255 had positive qualitative PCR results. Results of quantitative PCR on the same specimens demonstrated that in 14 patients, viral copy number per 10^5 cells had increased, pointing toward HCMV reactivation. In others, changes in viral copy number were mostly around 100/10^5 cells, with an upper limit of 300/10^5 cells.**

Conclusions: **Owing to the high prevalence of cytomegalovirus in our country, the chance of viral reactivation and HCMV infection/disease upon transplantation must be seriously considered. Therefore, use of quantitative PCR in PCR-positive patients is highly recommended to demonstrate active infection that may lead to HCMV disease during the posttransplant period. This also could help physicians begin pre-emptive therapy that would be for a shorter treatment period and provide for better outcomes in infected BMT patients.**

Key words: *Human cytomegalovirus, Bone marrow transplantation, Quantitative PCR, Viral load*

Human cytomegalovirus (HCMV) infection is the most common and potentially devastating opportunistic viral infection in bone marrow transplant (BMT) recipients, causing severe complications such as interstitial pneumonia, gastrointestinal disorders, and marrow suppression. Antiviral agents such as ganciclovir have clinical benefits, either preventing or curing HCMV disease after BMT [1-3]; however, because they are associated with toxicity and great expense, they must be used to treat only those patients with a high risk of HCMV disease. Thus, rapid and sensitive tests to diagnose HCMV infection are needed to predict or detect infection in the early stages of the disease before clinically significant symptoms occur. Serologic tests to detect specific anti-

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HCMV antibodies are not reliable markers of HCMV infection in BMT patients because of defective humoral responses and passively transferred antibodies [4-6]. In addition, viral cultures have weak sensitivity for showing HCMV disease during BMT.

Various techniques have been developed to address this limitation, including both nonmolecular and molecular tests. Because of its sensitivity, qualitative polymerase chain reaction (PCR) was the first method used, but this method cannot differentiate between infection and disease in transplant recipients. In contrast, quantitative PCR methods have shown that immune-suppressed patients with high viral loads are at great risk of developing HCMV disease [7-9].

The aim of this study, using previously developed qualitative and quantitative competitive PCR methods, was to detect and quantify HCMV genome load in peripheral blood mononuclear cells (PBMCs) of BMT recipients.

Materials and Methods

Twenty-six patients (17 males, 9 females; age range, 5-42 years; mean, 15.9 years) were admitted for BMT between April 2003 and September 2004 at the BMT Center of Namazi General Hospital in Shiraz, Iran. Three hundred thirteen blood samples were collected weekly from BMT patients as long as they were available. PBMCs from the patients were tested for HCMV by qualitative and quantitative PCR between 7 days before transplantation and 100 days after transplantation. One sample from each donor also was tested using qualitative and quantitative PCR (26 samples). Informed consent was obtained from all patients or their guardians.

Recipients did not receive ganciclovir prophylaxis. Patients with a high index of clinical suspicion of HCMV disease received ganciclovir only at the discretion of the clinicians. However, all patients received prophylactic acyclovir from the first week until at least 100 days after surgery.

Pretransplant HCMV IgG and IgM were tested in plasma samples of donors and recipients using qualitative ELISA kits (Dia.Pro Diagnostic Bioprobes, Milano, Italy) according to the manufacturer's instructions. Ten milliliters of whole blood was collected in a tube with ethylenediaminetetraacetic acid and processed within 4 hours after drawing. PBMCs were isolated using Ficoll-Hypaque density centrifugation at 1200 g for 30 minutes. Cells were then washed with phosphate-buffered saline (PBS) (pH, 7.4) and counted with a hematologic cell counter. PBMCs were adjusted with PBS to 10^6 cells/100 μL and frozen at -70°C until extraction. Plasma was harvested, aliquotted, and kept at -70°C.

DNA was extracted from 100 μL of cell suspension (10^6 cells) by digestion with 100 μg/mL protease K at 55°C for 1 hour. After boiling for 10 minutes, DNA was extracted with one volume of phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with 0.1 volume of sodium acetate (pH, 7.2) and 2 volumes of absolute ethanol. After centrifugation at 13 000 g for 30 minutes (Eppendorf centrifuge model number 5415R, Germany), the pellets were washed with 70% ethanol and resuspended in 50 μL of H_2O. A 5-μL sample was subjected to both qualitative and quantitative PCRs. Positive and negative controls for HCMV DNA were always included during the extraction procedure.

A modified qualitative PCR was performed [10]. For amplification, one set of primers (22 base primers, an upstream primer, 82494-82515 nucleotide position: 5´-3´ CGGTGGAGATACTGCTGAGGTC, and a down stream primer, 82729-82750 nucleotide position: 5´-3´ CAAGGTGCTGCGTGATATGAAG); full HCMV genome sequence (gene bank accession number: NC-001347) was used to generate the HCMV 257bp fragment, located within gB (glycoprotein B) (gene bank accession number: DQ089700). The 257 bp amplicons were generated using a Pfu enzyme in blunt-end form, purified, and then cloned into a PTZ57R plasmid. Amplification was carried out in 50 μL final volume containing 5 μL of extracted DNA, 1.5 U of Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 25 pmol of each primer, 10 mM tris-HCl, 50 mM KCl, 2 mM MgCl2, and 0.2 mM dNTP sets. PCR cycle parameters involved an initial incubation at 94°C, followed by 42 cycles at 94°C for 45 seconds, 60°C for 1 minute, and 72°C for 1 minute, with a final extension step of 10 minutes at 72°C (Eppendrof Mastercycler Gradient, Germany). The amplified DNA was separated by agarose gel electrophoresis. The gel was stained with ethidium bromide (2 μg/mL) for 10 minutes, washed, and photographed under UV illumination.

Quantitative PCR was done for specimens with positive qualitative PCR results. Target sequences were quantitated by competitive PCR with cloned internal standard (IS) sequences, which were produced by amplification of a 156 bp region of the lambda genome.

To achieve this, an HCMV primer binding site was added to the 5' end of lambda-specific primers. The result was a 200-bp IS sequence fragment (Table 1). Quantitative PCR was done by adding 1000 copies of IS to the PCR master mix, and the PCR cycle number was reduced to 38 instead of 42. Annealing temperature also was reduced from 60°C to 55°C. In each run, one series of competitive controls was tested; serial cloned gB DNA fragments were coamplified with 1000 IS copies. The 2 amplicons were separated by gel electrophoresis and UV visualized after ethidium bromide staining. Bands were quantified by Sion Image photo densitometry software (Version Beta 4.0.2, Sion Corporation, USA) to generate a standard curve by plotting the log ratio of gB/IS amplicons against the log of the gB copy number added to each PCR. The curve was used to interpolate the amount of HCMV DNA in clinical samples from their wild-type/competitor PCR product ratio.

A Pearson analysis was used to compare the correlation between IgM seropositivity, age, sex, and underlying disease with HCMV reactivation, and also ganciclovir therapy with a reduction in HCMV copy number (as a qualitative variable). A P value less than .05 was considered significant.

Results

Before transplantation, all 26 patients (100%) had anti-HCMV IgG, and 6 patients (20.3%) had anti-HCMV IgM antibodies. In donors, 25 (96%) had IgG, and 2 (7.7%) had anti-HCMV IgM (Table 2) antibodies respectively. After serial dilutions and preparation of the plasmid containing the HCMV 257 bp fragment, qualitative PCR was able to detect about 10 copies of the recombinant DNA. Quantitative PCR involving coamplification of 1000 IS copies could detect 10 to 10^5 plasmid copies. After coamplification of the clinical samples with 1000 IS copies, copies of less than 100 usually were not clearly visible on the gel. Therefore, samples positive in qualitative PCR but without a clear band in quantitative PCR were classified as having a viral copy number ≤ 100.

All patients were followed for 16 weeks, except for those traveling back to their hometown or those who passed away prior to the complete sample collection at 16 weeks. In total, there were 313 samples from 26 transplant recipients, of which 225 samples were positive for HCMV DNA. A majority of donor samples (18) also tested positive for HCMV DNA (copy number range: ≤ 100-150).

No significant relationship existed between age (P = .836), sex (P = .175), and the degree of reactivation of the virus. The relationship between the presence of IgM in donors (P = .492) or recipients (P = .914) and viral reactivation was not significant, as was the relationship between background disease and viral reactivation (P > .05).

HCMV-related symptoms were observed in 14 patients, and HCMV reactivation/infection was detected in 1 patient who did not develop HCMV disease. In patients with HCMV-related symptoms, on average, an increase in HCMV copy number was seen from the third week after transplantation, with the maximum copy number observed during the seventh week (Table 2). Ganciclovir treatment was started after an observation of HCMV clinical symptoms in the seventh week after transplantation, on average. In patients with fewer than 4300 viruses/10^5 cells, ganciclovir treatment reduced the copy number beginning at the first week of administration; however, in patients with copy numbers higher than 7000, it took 2 weeks to have an effect. In all cases, ganciclovir administration clearly was able to decrease the virus copy number but not to levels undetectable in PCR (Table 2).

Discussion

HCMV is one of the most important opportunistic infections in immunosuppressed patients, especially in organ transplant recipients [11-13]. Infection with HCMV leads to problems such as HCMV disease or graft rejection or a suppressed immune system, allowing other bacterial or fungal infections to invade the host [4].

Rapid diagnosis of active HCMV infection is important for beginning treatment, infection control, and reducing the progression and severity of the disease [9,14,15]. PCR and an antigenemia assay are

Table 1. The sequence and position of oligonucleotides used in production of the IS sequence

Oligonucleotide	Sequence (5'-3')	Position
Forward primer	CGGTGGAGATACTGCTGAGGTCTGCGTGTAGGCGAATTTG (22+18 mer)	40954-40971
Reverse primer	CAAGGTGCTGCGTGATATGAACCCACCGGAGAAACTAACGAC (22+20 mer)	41090-41109

Table 2. Demographic, virologic, and clinical characterizations of 26 BMT recipient patients with HCMV infection or disease

Patient No.	D/R Status*		Age	Sex	Underlying disease	Viral load		Ganciclovir (administration start week)	No. samples	No. positive PCR	Clinical presentation†
	IgG	IgM				Range	Lower after treatment				
1	+/+	-/-	8	M	Thalassemia	≤ 100	-	No	7	2	-
2	-/+	-/-	38	M	AML	≤ 100 - 135	-	No	14	6	-
3	+/+	-/-	8	M	Thalassemia	≤ 100	-	No	7	2	-
4	+/+	-/-	18	M	ALL	≤ 100	-	No	7	3	-
5	+/+	-/-	8	F	Thalassemia	≤ 100 - 4300	300	Yes (6)	15	13	Gastroenteritis + GVHD
6	+/+	-/-	12	M	Thalassemia	≤ 100	-	No	14	5	-
7	+/+	-/-	7	M	Aplastic anemia	≤ 100 - 1450	700	Yes (4)	7	6	Gastroenteritis + hepatitis + GVHD
8	+/+	-/-	6	M	Thalassemia	≤ 100	-	No	9	5	-
9	+/+	-/+	10	M	Thalassemia	≤ 100 - 300	≤ 100	Yes (5)	12	9	Renal involvement +fever+GVHD
10	+/+	+/-	8	M	Thalassemia	≤ 100	-	No	16	9	-
11	+/+	+/-	33	F	AML	≤ 100 - 6500	-	No	13	12	Gastroenteritis + GVHD
12	+/+	-/-	8	M	Thalassemia	≤ 100 - 1200	≤ 100	Yes (8)	16	12	pneumonitis
13	+/+	-/-	5	M	Thalassemia	≤ 100 - 8500	450	Yes (7)	16	14	Gastroenteritis + GVHD‡
14	+/+	-/-	31	M	AML	≤ 100 - 15200	8100	Yes (6)	11	9	pneumonitis
15	+/+	-/-	14	M	Aplastic anemia	≤ 100	-	No	11	3	-
16	+/+	/-	17	F	AML	≤ 100 - 1600	700	Yes (6)	13	11	Gastroenteritis + renal involvement
17	+/+	-/-	21	F	AML	≤ 100	-	No	9	5	-
18	+/+	-/+	19	M	AML	≤ 100 - 2200	380	Yes (7)	12	10	Renal involvement + GVHD‡
19	+/+	-/+	8	F	Thalassemia	≤ 100 - 150	No	11	8	-	
20	+/+	-/+	22	M	CML	≤ 100 - 2800	200	Yes (5)	14	13	Pneumonitis
21	+/+	-/-	21	F	AML	≤ 100 - 3500	500	Yes (7)	16	13	renal involvement + fever
22	+/+	-/+	10	M	Thalassemia	≤ 100 - 900	-	No	13	11	Gastroenteritis + renal involvement + GVHD
23	+/+	-/-	12	F	Thalassemia	≤ 100 - 7000	670	Yes (6)	16	16	Gastroenteritis + renal involvement + hepatitis
24	+/+	-/-	42	F	AML	≤ 100 - 250	-	No	8	7	-
25	+/+	-/-	16	M	Thalassemia	≤ 100 - 2600	≤ 100	Yes (6)	15	14	Gastroenteritis + GVHD
26	+/+	-/+	11	F	Thalassemia	≤ 100 - 340	-	No	11	8	-

AML: Acute myeloid leukemia, BMT: Bone marrow transplant, CML: Chronic myeloid leukemia, GVHD: Graft versus host disease, HCMV: Human cytomegalovirus, M: Male, F: Female

*D: Donor, R: Recipient

†Only HCMV-linked clinical symptoms are cited in this table

‡mild GVHD

common methods for detecting HCMV infection [16, 17]. Quantitative PCR can measure changes in virus copy number and has a higher positive predictive value than does qualitative PCR in different patient populations suspected of having HCMV disease. It is a more appropriate test than antigenemia in BMT patients owing to the low white blood cell counts in this population. This is especially true in societies with a high prevalence of HCMV infection [18-21]. Monitoring patients and treatment decisions are much easier for D-/R- or D+/R- patients rather than D+/R+ or D-/R+ because the latter group requires more than a positive PCR test result to start treatment (D = donor, R = recipient, + = seropositive, - = seronegative) [4]. All of the recipients, and all but 1 of the donors had been infected previously with cytomegalovirus (Table 2). Under these circumstances, such BMT recipients should be considered at a high risk of latent HCMV reactivation and followed for posttransplant HCMV infection/disease.

In this study, HCMV disease was diagnosed by clinical symptoms and virologic confirmation of HCMV infection (nested PCR on plasma, data not shown). The average age of the transplant recipients was 12 years old because most recipients were thalassemic children. Of the 14 patients that showed symptoms of involvement with HCMV, 8 (57%) had gastroenteritis, and 4 had additional symptoms such

as hepatitis and renal involvement. Renal involvement with fever, with or without hemorrhagic cystitis, occurred in 3 patients, and pneumonia occurred in 3 others.

In the population studied, antiviral treatment with ganciclovir was started only after clinical symptoms of HCMV disease appeared; in most patients, when the virus had its maximum copy number in 10^5 cells (Table 2). To prevent the disease and limit its problematic consequences more effectively, it is recommended that anti-HCMV treatment be given prophylactically to patients demonstrating an increasing trend in their viral load. The promising fact in the treatment of these patients is that after ganciclovir administration, viral copy number decreased, even though it did not reach undetectable levels in PBMCs. This can be explained by the fact that these cells are the main site of HCMV latency.

Comparing the quantitative PCR results from patients affected by HCMV, with those that were not affected by HCMV, as well as those of donors, it seems that 300 virus copies in 10^5 cells could be considered as the cut-off value in our test.

The results of the current study demonstrate that there is a 2- to 4-week gap between HCMV reactivation/infection commencement and manifestation of HCMV disease. This interval lets the physician use current molecular viral diagnostic techniques to make accurate decisions on treatment. At our center, using quantitative competitive PCR seems to be helpful in deciding whether or not to treat with ganciclovir by estimating the virus copy number in BMT recipients.

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