Qualitative and Quantitative Detection of Cytomegalovirus DNA in Sera by PCR as a Clinical Marker

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The amount of human cytomegalovirus (CMV) DNA in sera is considered to be a direct marker for CMV infection. We established conditions for nested PCR that detected one copy of CMV DNA, and for competitive PCR, which detected five or more copies of CMV DNA quantitatively. We tested 50 μ l each of 16 freeze-stored and 5 fresh sera from patients, for CMV DNA. In sera obtained from the same patient at different time points, small amounts of CMV DNA were detected before the onset of CMV pneumonia. In sera from certain CMV-infected patients who were treated with the anti-CMV agent, ganciclovir, CMV DNA was not detected. Quantitative PCR detection of CMV DNA seems to be suitable for predicting early recurrent CMV infection and monitoring the efficacy of antiviral therapy. The qualitative nested PCR examination of CMV DNA in 40 cord blood plasma samples was carried out for the purpose of preventing CMV infection by cord blood stem cell transplantation, and they were all negative for CMV DNA.

Key words serum cytomegalovirus (CMV) DNA; qualitative PCR; quantitative PCR; competitive PCR; cytomegalovirus (CMV)-associated disease

Although latent infection by cytomegalovirus (CMV) in leucocytes is observed in many Japanese, clinical symptoms rarely develop. However, CMV is activated under conditions resulting in decreased immunocompetence, such as AIDS, leukemia, malignant lymphoma and immunosuppressant administration after organ transplantation, and various organs and tissues are recurrently infected with CMV. Although diagnosis of CMV infection has usually been established by viral isolation or serum antibody examination, viral culture requires considerable time. Anti-CMV antibody titers do not act as direct markers of viremia. Moreover, antibodies against CMV are detected in healthy individuals who are latently infected by CMV. Therefore, diagnosis of CMV infection by serum antibody examination is not reliable. The shell vial assay^{1,2)} and the use of monoclonal antibody that recognizes p65 antigen in CMV,³⁾ are improved methods for early diagnosis of CMV infection. However, tissue culture is required in both methods. Detection of CMV from the organs or tissues is preferable for definitive diagnosis of recurrent CMV infection. However, it is difficult to perform biopsy on patients with constitutional symptoms, due to decreased immunocompetence. For early diagnosis of CMV infection as well as evaluation of therapeutic responses, quantitative detection of activated CMV should be performed safely, rapidly and reliably without any burden on the patient. Activated CMV are abundant in urine samples from patients with CMV infection. Therefore, qualitative detection of urinary CMV DNA using PCR was developed.⁴⁾ However, since renal functions vary depending on the condition of the patient, urine samples are not suitable for quantitative detection of CMV DNA. Qualitative PCR detections of CMV DNA from blood or plasma have also been reported from many laboratories. In the present study, using competitive PCR, we have quantitatively detected CMV DNA in freeze-stored sera obtained from patients suspected of CMV infection as well as AIDS patients who are prone to CMV infection, and investigated whether quantitative CMV detection could be used as an

index for early diagnosis of recurrent CMV infection, predicting changes in the patient's condition and evaluating therapeutic responses.

MATERIALS AND METHODS

Samples Serum from healthy individuals, in which no CMV DNA was detected by nested PCR and anti-CMV antibody was negative in the enzyme-linked immunosorbent assay (anti-IgM and anti-IgG) and in complement fixation test, was used as negative control. Positive controls were prepared by adding certain copies of standard CMV DNA (Custom CMV DNA Extract PCR Standard, Advanced Biotechnologies Inc., Columbia, MD, U.S.A.) or competitor DNA to negative control sera. We used 16 samples of freeze-stored (-80°C) sera obtained from 11 patients, 5 samples of fresh sera from 2 patients and 40 samples of cord blood for stem cell transplantation. These samples were provided by Shizuoka Children's Hospital.

PCR Primers A pair of PCR primers (MIE-4 and MIE-5)⁴⁾ were used to amplify the 435 bp DNA fragment contained in the major immediate early (MIE) gene⁵⁾ of CMV. In the CMV gene (total length: approximately 235 kbp), the MIE gene is transcribed to mRNA immediately after palindromic CMV infection. Another pair of primers (IE-1 and IE-2)⁶⁾ were used for nested PCR to partially amplify the 159 bp DNA fragment contained in the initial PCR product.

Qualitative PCR of CMV DNA We evaluated various PCR conditions using a GeneAmp PCR System 9600 (Perkin-Elmer Co., Norwalk, CT, U.S.A.) and the final concentrations of each reagent, primer and enzyme contained in 50 μ l of reaction mixture were determined as follows: MgCl_2 : 2.5 mM, deoxynucleoside triphosphates (dNTPs): 200 μM each, primer: 0.5 μM each, and AmpliTaq Gold (PE Japan Applied Biosystems, Chiba, Japan): 1.25 units. After preheating the reaction mixture at 94°C for 12 min to activate the enzyme, PCR was carried out as follows: denatura-

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Fig. 1. Competitive PCR of Standard CMV DNA in Sera

Different amounts of standard CMV DNA and 100 copies of competitor DNA in sera were extracted and amplified in the same tube using MIE-4/MIE-5 primer pair for 50 cycles. M is a ladder size marker.

tion at 94 °C for 40 s, annealing at 65 °C for 30 s, and elongation at 72 °C for 25 s. The initial PCR was repeated for 50 cycles, while nested PCR was repeated for 45 cycles using 5 μ l of the initial PCR product, and the respective PCR cycles were followed by additional elongation at 72 °C for 6 min. After agarose/ethidium bromide gel electrophoresis of 10 μ l of each amplified product, the DNA band was detected.

Competitor DNA Competitor DNA, an internal standard, was prepared using PCR MIMIC Construction Kit (Clontech Lab., Palo Alto, CA, U.S.A.) according to the manufacturer's instructions. Using MIE-4/MIE-5 primer pair, an amplified competitor DNA fragment (270 bp) was obtained.

Competitive PCR of CMV DNA Ten-fold diluted competitor DNA and 10 μ g carrier glycogen were added to 50 μ l freeze-stored sera. After treatment with Proteinase K (E. Merck, Darmstadt, Germany), CMV DNA and competitor DNA were extracted and purified by the usual phenol/chloroform/isoamyl alcohol method. PCR was performed as described above for 45 cycles. After agarose/ethidium bromide gel electrophoresis, the DNA concentration in each band was compared and quantified using a Densitograph (model AE-6900 M, Atto Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Conditions for Detecting One Copy of CMV DNA

CMV DNA was extracted and purified from 50 μ l of anti-CMV antibody-negative and CMV DNA free sera treated with 0–1000 copies of standard CMV DNA, and various conditions for qualitative CMV DNA detection by PCR were evaluated using the MIE-4/MIE-5 primer pair. As a result, under the conditions described in Materials and Methods, one copy of CMV DNA was detected by the initial PCR (data not shown). Then, CMV DNA was detectable qualitatively from patients' sera by the initial PCR alone. When negative results were obtained from the initial PCR, nested PCR was further performed twice to examine CMV DNA in the sera at a higher sensitivity.

Competitive PCR of CMV DNA We extracted and purified DNA from 50 μ l sera with 0–1000 copies of standard CMV DNA, added 100 copies each of competitor DNA, and performed competitive PCR. Two bands of amplified DNA fragments with almost identical densities on electrophoresis were observed when 100 copies of standard CMV DNA and competitor DNA were added to the sera (Fig. 1). The density of the amplified competitor DNA fragment was slightly

lighter than that of the amplified standard CMV DNA. This was most probably due to the lower number of bases in amplified competitor DNA than in amplified standard CMV DNA, but this was not a problem for practical use. The lower limit for quantitative CMV DNA detection by competitive PCR was 5 copies.

Qualitative Detection of CMV DNA in Sera from Patients Qualitative detection of CMV DNA was performed by PCR using 50 μ l each of freeze-stored sera, as shown in Table 1 (No. 1–16). Amplified CMV DNA fragments (435 bp) were electrophoretically detected in 6 samples (serum no. 1, 4, 7, 8, 10 and 12), and these samples were concluded to be positive for CMV (Fig. 2A). The remaining 10 samples (serum no. 2, 3, 5, 6, 9, 11, 13, 14, 15 and 16), in which amplified CMV DNA fragments were not electrophoretically detected, were tested further by nested PCR using 5 μ l of each amplified product from the initial PCR, but no amplified CMV DNA fragments (159 bp) were detected. These samples were concluded to be negative for CMV.

Quantitative Detection of CMV DNA in Sera from Patients by Competitive PCR Using 6 samples evaluated as positive for CMV DNA by qualitative PCR, quantitative CMV DNA detection was performed using competitive PCR. In sera no. 4, 7 and 10, in which concentrations of CMV DNA were low, CMV DNA was not sufficiently amplified in the presence of competitor DNA. The electrophoretic profiles of the competitive PCR of sera no. 1, 8 and 12 were densitometrically compared with those of the competitor DNA, and the levels of CMV DNA in 50 μ l sera were calculated as 617, 10 and 397 copies, respectively (Fig. 2B).

Correlation between the Amount of CMV DNA in Sera and Patient's Symptoms Table 1 summarizes the correlation between the results of CMV DNA detection by PCR and patients symptoms. Serum no. 1 was from a patient with malignant lymphoma complicated by CMV pneumonia, due to decreased immunocompetence, and 617 copies of CMV DNA were detected in 50 μ l of this sample.

Sera no. 2 to 4 were from the same patient collected at different time points. This patient underwent bone marrow transplantation (BMT) on January 12, 1995. In the patient's pre-BMT serum collected on January 5, anti-CMV antibody was positive but CMV DNA was not detected (Table 1). CMV DNA was detected in the serum obtained 40 d after BMT, but the concentration was below the lowest limit of detection by competitive PCR (5 copies) (Fig. 3A). This patient died in early April following the onset of CMV pneumonia, and it was concluded that recurrent CMV infection resulting

Table 1. Detection of CMV DNA in Sera and Patients' Serological and Clinical Status

Serum no.	PCR		Serological status ^{b)}			Clinical status
	Qualitative	Quantitative ^{c)}	Anti-IgM	Anti-IgG	CF test	
1	+	617	+ (3.67)	NT	+ (X32)	Malignant lymphoma, CMV pneumonia
2	– (95.1.5.)	5> ^{d)}	– (0.80>)	+ (53.1)	+ (X32)	Same patient, BMT ^{c)} (95.1.12.), malignant lymphoma, CMV pneumonia (early April 95)
3	– (95.2.9.)		– (0.80>)	NT	+ (X32)	
4	+ (95.2.22.)		– (0.80>)	+ (80.8)	+ (X32)	
5	–		– (0.80>)	± (3.1)	– (X4>)	Nephroblastoma, interstitial pneumonitis
6	–	5>	– (0.80>)	+ (25.6)	+ (X16)	Acute lymphemia, interstitial pneumonitis
7	+		– (0.80>)	+ (67.3)	+ (X64)	Aplastic anemia
8	+		NT	NT	+ (X64)	AIDS, CMV cardiomyopathy, MAC ^{e)}
9	–		NT	+ (8.5)	NT	AIDS, CMV retinitis, AIDS dementia complex, progressive multifocal leucoencephalitis
10	+	5>	NT	NT	+ (X16)	AIDS, CMV retinitis, MAC
11	–	397	NT	+ (128.0)	NT	Same patient, AIDS, CMV pneumonia (early March 91)
12	+		– (0.80>)	+ (56.3)	NT	
13	– (90.6.4.)		NT	NT	– (X4>)	
14	– (90.12.7.)		NT	NT	– (X4>)	
15	– (91.1.18.)	12	NT	NT	– (X4>)	Same patient, BMT (96.12.3.)
16	– (91.2.14.)		NT	NT	– (X4>)	
17	– (96.12.27.)		– (0.80>)	+	NT	
18	+ (97.1.13.)		– (0.80>)	+ (52.4)	+ (X8)	
19	+ (97.1.27.)	44	NT	+	NT	Same patient, CMV pneumonia
20	+ (97.12.8.)		–	+	NT	
21	– (97.12.24.)		–	+	NT	

a) CMV DNA copies/50 μ l serum. b) CMV IgM and IgG antibody titers were measured by enzyme-linked immunosorbent assay, CF: complement fixation, NT: not tested. c) Bone marrow transplantation. d) Less than the quantitative detection limit by competitive PCR (5 copies). e) *Mycobacterium avium* complex.

Fig. 2. Qualitative and Quantitative Detection of CMV DNA in Patient Sera by PCR

CMV DNA in 50 μ l freeze-stored sera from patients (lanes 1 to 16) were amplified qualitatively (A). CMV DNA in 50 μ l sera which showed positive results in the qualitative PCR were quantified by competitive PCR (B). N and P are negative and positive (100 copies standard CMV DNA) controls, respectively. Quantitative detection limit of CMV DNA is 5 copies.

Fig. 3. Time Course Quantitative Detection of CMV DNA in Patient Sera

CMV DNA in 50 μ l freeze-stored sera (A) and fresh sera (B) from patients who underwent BMT were detected quantitatively by competitive PCR.

from reactivation of latent CMV caused the death of this patient, since latent CMV is known to be reactivated by decreased immunocompetence due to immunosuppressant administration after BMT. It has been reported that CMV pneumonia reaches its crisis peak at 2–3 months after BMT.⁷⁾ This example showed that time course determination of serum CMV DNA levels was useful for early diagnosis and treatment of recurrent CMV infection.

Sera no. 5 and 6 were from patients with interstitial pneumonia. Since CMV DNA was not detected in these sera, it was deduced that the pneumonia in these patients was not caused by CMV infection. No anti-CMV antibody was detected from serum no. 5 (Table 1).

Serum no. 7 was from a patient with aplastic anemia. CMV DNA was below the limit of quantitative detection. Serum no. 12 was from an AIDS patient, and 397 copies of CMV DNA were detected in 50 μ l of this serum sample (Fig. 2B). Both cases suggest CMV infection developing in these patients. These results indicated the possibility that serum CMV DNA detection was useful in the early diagnosis and treatment of CMV infection.

Sera no. 8 through 11 were from 4 AIDS patients who were diagnosed as complicated by CMV infection. However, CMV DNA was not detected in 2 samples (sera no. 9 and 11). Although sera no. 8 and 10 were positive for CMV DNA detection by PCR, the levels of CMV DNA in 50 μ l of serum sample were 10 copies, or below the limit of quantitative detection (Fig. 2B). It was reported that CMV DNA was not detectable in plasma when patients were successfully treated with the CMV DNA polymerase inhibitor, ganciclovir.^{8–12)} Since ganciclovir was administered to these 4 patients, it is reasonable that detection of CMV DNA was difficult in these serum samples. The results obtained with these serum samples suggests that quantitative detection of serum CMV DNA levels with time is useful for evaluating therapeutic responses.

Sera no. 13 through 16 were from the same AIDS patient who later died of CMV pneumonia. These sera were collected at different time points over 8 months before the onset of CMV pneumonia. However, all sera were negative for CMV DNA, although a serum sample was not obtained from this patient immediately after the onset of CMV pneumonia. It seemed that CMV DNA remained undetectable in the blood before the onset of pneumonia.

Clinical Use of CMV DNA Detection In this report, we examined comparatively the results of CMV DNA detection in freeze-stored sera obtained from patients and their symptoms. Several recent reports^{10–14)} have shown the efficacy of PCR for clinical management of CMV infection. The following cases demonstrated the clinical usefulness of CMV DNA detection.

In the first case (sera no. 17 to 19) in which a patient underwent BMT on December 3, 1996, we demonstrated increased levels of CMV DNA in the sera over time, as a clinical marker of CMV infection (Fig. 3B). Although CMV DNA was not detected in serum obtained on December 27, 24 d after BMT, 12 copies of CMV DNA were detected in 50 μ l of the serum sample obtained on January 13, 1997, 41 d after BMT. On January 27, 1997, antiviral therapy with ganciclovir was initiated since the level of CMV DNA had increased to 44 copies per 50 μ l serum.

The second case (sera no. 20 and 21 in Table 1) was a patient with pneumonia, admitted as an emergency on December 8, 1997. CMV pneumonia was suspected by serum antibody examination (sero-positive for anti-CMV IgG and complement fixation test). Qualitative PCR was immediately performed, and an amplified CMV DNA fragment was electrophoretically detected. Therefore, ganciclovir administration was initiated. As a result, CMV DNA was not detected in the serum obtained on December 24, 1997, and the prognosis of this patient was satisfactory (data not shown).

The third case involved application of the CMV DNA test

in the prevention of CMV infection during cord blood stem cell transplantation. Using 40 frozen cord blood plasma samples, qualitative CMV DNA detection was performed by PCR. The initial PCR using the MIE-4/MIE-5 primer pair demonstrated that all samples were negative for CMV DNA. Therefore, nested PCR using another primer pair (IE-1/IE-2) was performed. All the samples were negative for CMV DNA. We intend to perform CMV DNA detection on all cord blood samples to be used for stem cell transplantation hereafter, because it has been suggested that CMV is present in approximately 0.1% of cord blood (personal communication).

The MIE gene⁵⁾, partially amplified by PCR in this study, is the gene expressed immediately after initial or recurrent CMV infection, and serves as a suitable marker for early diagnosis of recurrent CMV infection. Thus, quantitative analysis of the mRNA of the MIE gene by competitive reverse transcription (RT)-PCR may serve as well, or better for definitive and quantitative verification of recurrent CMV infection.

Acknowledgements We thank Mr. Takashi Kurihara for his technical assistance in this experiment. This work was partly supported by a Grant-in-Aid for Scientific Research from the Shizuoka Prefectural Foundation for the Advancement of Education, Japan.

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