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Detection of Human Cytomegalovirus Immediate Early Gene-Specific mRNA by Reverse Transcription Polymerase Chain Reaction.

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ABSTRACT **We have established a method for detection of human cytomegalovirus (HCMV) immediate early gene (IE)-specific mRNA by reverse transcription polymerase chain reaction (RT-PCR). Using the IE-1 specific primer fragments designed for RT-PCR, we have amplified a 232 base-pair fragment which represents IE-1 mRNA transcribed from the IE-1 gene of HCMV AD169 strain. No DNA cross reactivities to other human herpes virus DNAs or human embryonic lung cell (MRC-5 line) DNA were observed. The RT-PCR assay indicated HCMV IE-1-specific mRNA in HCMV-infected MRC-5 cells and in peripheral blood specimens from one of 3 patients examined. The results indicate that RT-PCR will be readily applicable to rapid detection of HCMV mRNA and may be useful for diagnosis of active primary or recurrent HCMV infections.**

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Key words: Human cytomegalovirus, RT-PCR, Immediate early gene, mRNA

Introduction

Human cytomegalovirus (HCMV) usually causes mild or asymptomatic infections in healthy individuals. However, the virus is a major pathogen in immunocompromised hosts including those with an acquired immunodeficiency syndrome and is a common infectious cause of congenital abnormalities (1, 2). Allogenic bone marrow transplant (BMT) recipients are at a particular risk of disseminated HCMV disease with interstitial pneumonia (3). The diagnosis of HCMV infection is clinically difficult and usually depends on isolation of the virus by tissue culture (4), which requires up to 4 weeks. Recently, a method employing the polymerase chain reaction (PCR) has been developed and applied to detect HCMV genome DNA (5, 6, 7). PCR makes it possible to detect HCMV DNA obtained from clinical specimens within a few hours and with greater sensitivity than tissue culture. However, the investigation of viral DNA expression is essential for diagnosis of persistent as well as acute HCMV infection. Reverse transcription polymerase chain reaction (RT-PCR) is a powerful search tool for identification of the expression of viral genes (8). In this study, we have established an RT-PCR method for rapid and sensitive detection of HCMV immediate early gene-(IE-1)-specific mRNA. The expression of IE-1 gene was also comfirmed in tissue culture cells infected with HCMV and in clinical specimens.

Materials and Methods

Virus and cell

Human embryonic lung cell line (MRC-5) was maintained in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (Flow Lab., McLean, VA). HCMV strain AD169 was obtained from the American Type Culture Collection (Rockville, MD) and passaged in MRC-5 cells according to the standard tissue culture technique (9).

Clinical specimens

Six heparinized peripheral blood samples and urine specimens were obtained from 3 patients: 2 patients with hepatitis and one with mononucleosis. A buffy coat was prepared by the following protocol. Heparinized peripheral blood (5 m*l*) was lysed with 10 volumes of 0.2% NaCl and centrifuged at 2500 rpm for 10 min. Pellets were then subjected to DNA extraction.

Virus isolation from urine

HCMV from urine specimens was isolated according to the standard tissue culture technique (9). The presence of HCMV was confirmed by observation of its characteristic cytopathic effect (CPE), and by immunofluorescent staining with monoclonal antibodies raised against the HCMV IE antigen (Ortho Diagnostics Systems, Carpinteria, CA) (10).

Viral and cellular DNA

The following viral and cellular DNAs were used for templates of PCR: Herpes simplex virus-1 (HSV-1, F strain), Herpes simplex virus-2 (HSV-2, G strain), Varicella-Zoster virus (VZV, Oka strain), Epstein-Barr virus (EBV)-infected Raji cells, Human herpes virus-6 (Hashimoto strain), Human cytomegalovirus (HCMV, AD169 and Davis strains) and Human embryonic lung cell (MRC-5 line). Viral and cellular DNA extractions were carried out according to the standard technique (11). Cells infected with HCMV were initially lysed with a buffer (10 mM Tris-HCl pH 7.4, 10 mM disodium EDTA, 0.6% sodium dodesyl sulfate) containing 50 μg/m*l* of proteinase K (Wako Co., Japan). Viral and cellular DNAs were successively extracted from cell lysates twice with equal volumes of phenol -chloroform and finally with chloroform-isoamylalcohol (24 : 1). The DNA in aqueous phase was precipitated with ethanol.

Preparation of whole cell RNA

MRC-5 cells were infected with HCMV AD169 strain at multiplicities of infection (moi) of 0.5 plaque formation unit (pfu) per cell. Cells were harvested with a rubber scraper at 24 hours (hrs) after infection and washed twice with phosphate-buffered saline (PBS). Total RNA was extracted from HCMV-infected MRC-5 cells and a buffy coat of peripheral blood with guanidine thiocyanate (GTC) (12). Briefly, cell pellets were suspended and lysed in 2 m*l* of GTC buffer (4 M Guanidine thiocyanate, 25 mM sodium acetate pH 6.0, 1% 2-mercaptoethanol) and laid on top of a CsCl buffer (5.7 M CsCl, 0.1 M EDTA pH 7.5). Samples were centrifuged at 33,000 rpm for 18 hrs at 20℃ using a Beckman SW41 rotor. RNA was recovered by ethanol-precipitation.

Oligonucleotide primers for RT-PCR

Using a computer homology search system, the following oligonucleotides were designed for RT-PCR as a primer: PIE-1 ($^{5'}$CAAGAGAAAGATGGACCCTGAT$^{3'}$ nucleotides 980-1001) and PIE-2 ($^{5'}$CAGGACATCTTTCTCGGGGTTC$^{3'}$ nucleotides 1304-1325). Their positions on the HCMV AD169 genome are diagrammed in Fig. 1. These oligonucleotides were synthesized with a DNA synthesizer Model 7500 (Milligen, Corp., Redford, MA) and purified by high pressure liquid chromatography (HPLC).

Reverse transcription

Each 30 μl reaction mixture contained 1X PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mg/ml nuclease-free bovine serum albumin), 1 mM of dNTP, 65 units of human placental ribonuclease inhibitor (TaKaRa Shuzo Co., Ltd., Japan), 100 picomoles oligo dT primer (12-18 nucleotides), 1-10 μg of total RNA and 10 units of Rous associated virus-2 reverse transcriptase (TaKaRa). After incubation at 42℃ for 60 min, reactions were stopped by heating at 94℃ for 10 min.

PCR

The PCR method was based on that described by Saiki et al (13). Each 100 μl reaction mixture contained 1/10 volume of the reverse transcription product, 10 mM This-HCl pH 8.3, 50 mM KCl, 0.1% Gelatin, 1.5 mM $MgCl_2$, 1 mM of dNTP, 0.05 μM each of PIE-1 and PIE-2, and 2.5 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of denaturation at 94℃ for 1 min, annealing at 55℃ for 2 min and extension at 72℃ for 2 min. The 30 cycles were performed with DNA Thermal Cycler (Perkin-Elmer Cetus). PCR products were electrophoresed through 3% Nusieve and 1% Seakem agarose gels (FMC Corp., Rockland, ME), stained with 1 μg/ml ethidium bromide and visualized with an ultraviolet transilluminator. Sizes in base-pair (bp) were determined by mobilities relative to standard DNA size markers: HincII digests of ΦX174 RF DNA (TaKaRa) and 1 Kb ladder (Bethesda Res. Lab., Gaithersburg, MD). For the Southern blot hybridization assay, DNA was transfered to a nylone filter, incubated with HCMV strain AD169 DNA labeled with digoxygenin, and detected with the Nucleic Acid Detection Kit (Boehringer Mannheim Yamanouchi Co., Ltd., Germany).

Results

Primer design and characterization of amplified products

The HCMV IE-1 gene is indispensable for virus production (14). Primers were designed for RT-PCR analysis of the IE-1 gene-specific mRNA. We selected the IE-1 nucleotide sequences, which, according to a computer search, have low homologies to other herpes virus DNAs. The HCMV IE-1 gene has three introns and four exons (Fig. 1-C). The PIE-1 and PIE-2 primers designed for RT-PCR were located on the second and third exons of IE-1, respectively. The amplified fragment from mature mRNA should be 232 bp long without the second intron; the amplified fragment from HCMV genome DNA should be 346 bp long including the intron. Thus, the primers have been designed to distinguish amplifications derived from DNA or RNA tem-

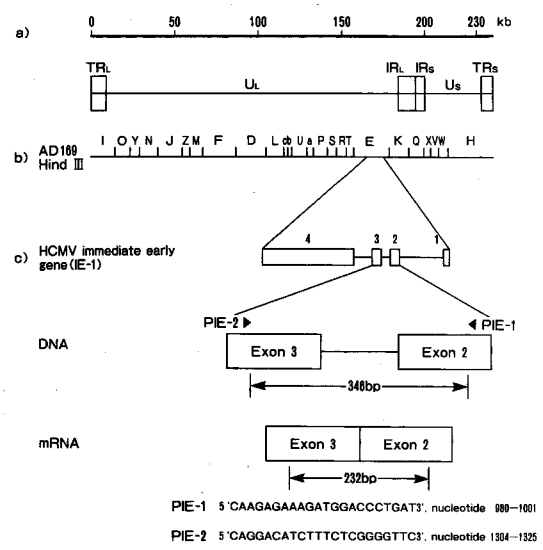

Fig. 1 Locations of the RT-PCR primers on the immediate early gene region of HCMV AD169
a) Structural arrangement of HCMV genome
The genome consists of a long (L) and short (S) component. Sequences in each unique (U) region are bounded by terminal repeats (TR) and inverted internal repeats (IR).
b) Restriction map of HCMV AD169 strain DNA with Hind III
c) Locations of PIE-1 and PIE-2 primers
Diagram of structure of HCMV AD169 immediate early gene (IE-1) are shown based on the nucleotide sequences determined by Akrigg *et al* (24). Boxes with numbers denote exons and lines between boxes show introns. The PIE-1 and PIE-2 primers are mapped at the second and third exons, respectively.

plates. Total RNA extracted from HCMV-infected cells 24 hrs post-infection was analyzed by RT-PCR using these primers (Fig. 2). We detected two bands by electrophoresis of RT-PCR products (Fig. 2, lane 2). To characterize these bands, we treated total RNA with either DNase I or RNase A before reverse transcription reactions. By digestion with RNase A, an upper fragment band of 346 bp was generated (Fig. 2, lane 3); in contrast, only a lower fragment of 232 bp was generated by DNase I digestion (Fig. 2, lane 4). Both fragments were hybridized with HCMV DNA, as shown by Southern blot hybridization (lower panel in Fig. 2). Thus, the amplified fragment of 232 bp must have been processed from HCMV-specific RNA. The fragment of 346 bp was apparently an amplification product derived from traces of HCMV DNA which was co-extracted with total cell RNA. The results showed that HCMV IE-1-specific mRNA was detectable with RT-PCR using the PIE-1 and PIE-2 primers.

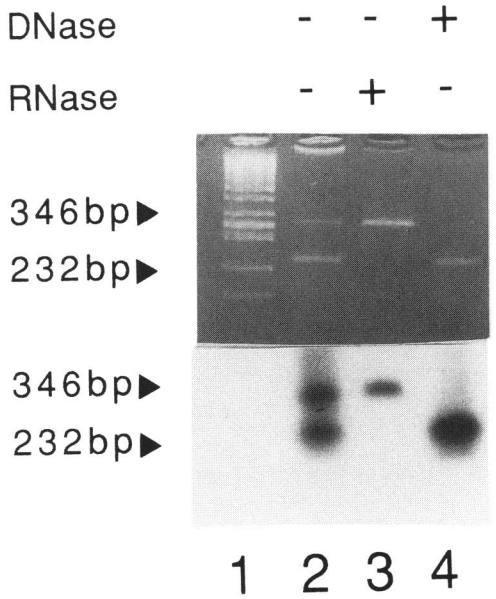

Fig. 2 Characterization of products amplified by RT-PCR
Total RNA was extracted from HCMV-infected MRC-5 cells (moi: 0.5 pfu per cell) 24 hrs post-infection and treated with either RNase A (50 $\mu g/ml$, Worthington Biochemicals, Freehold, NJ lane 3) or DNase I (20 units/ml, Promega Corp., Madison, WI lane 4) for 10 min at 37℃ RT-PCR was carried out using the PIE-1 and PIE-2 primers. Total RNA without nuclease treatments was also analyzed (lane 2). Amplified products were separated by 3% NuSieve and 1% Seakem agarose gel and stained with ethidium bromide (upper panel). Amplified products were analysed by Southern blot hybridization using HCMV DNA probe (lower panel). Lane 1: Size marker, HincII fragment of Φ X174 DNA

Specificity and sensitivity of amplification using designed primer pairs

Specificity of amplifications was determined by using each viral DNA of HCMV, HSV-1, HSV-2, VZV, EBV, and HHV-6, as well as cellular DNA from human embryonic lung cells (MRC-5) as templates for PCR. Products amplified by PCR were electrophoresed, stained, and photographed as shown in Fig. 3-a. The DNA fragment with an expected size of 346 bp was obtained with both AD169 and Davis strains of HCMV (Fig. 3-a, lanes 2 and 3), but not with HSV-1, HSV-2, VZV, HHV-6, and MRC-5 DNA (Fig. 3-a, lanes 4-9). These data indicated that the PIE-1 and PIE-2 primers used here were highly specific for HCMV DNA. Next, we analyzed the sensitivity of PCR. The plasmid pAD346,

Table 1 *Detection of HCMV IE-1 DNA and mRNA in clinical specimens*

Patients	Age	Sex	Diagnosis	acute phase				convalescent or chronic phase			
				Virus isolation[1]	ELISA (IgM)[2]	DNA[3]	mRNA[3]	Virus isolation[1]	ELISA (IgM)[2]	DNA[3]	mRNA[3]
1	7 M	M	Hepatitis	+	+	+	+	ND	ND	+	−
2	10 M	M	Mononucleosis	+	+	+	−	+	−	+	−
3	9 M	M	Hepatitis	+	+	+	−	ND	ND	+	−

+: positive, −: negative ND: not determined

[1] Virus isolation from urine was done as described(4)

[2] Serum sample were analyzed by ELISA using IgM antibody raised against HCMV early and late antigen (LA): positive≧0.20 OD, negative<0.20 OD, OD: optical density(23)

[3] DNA and mRNA from buffy coats were assayed by PCR and RT-PCR, respectively.

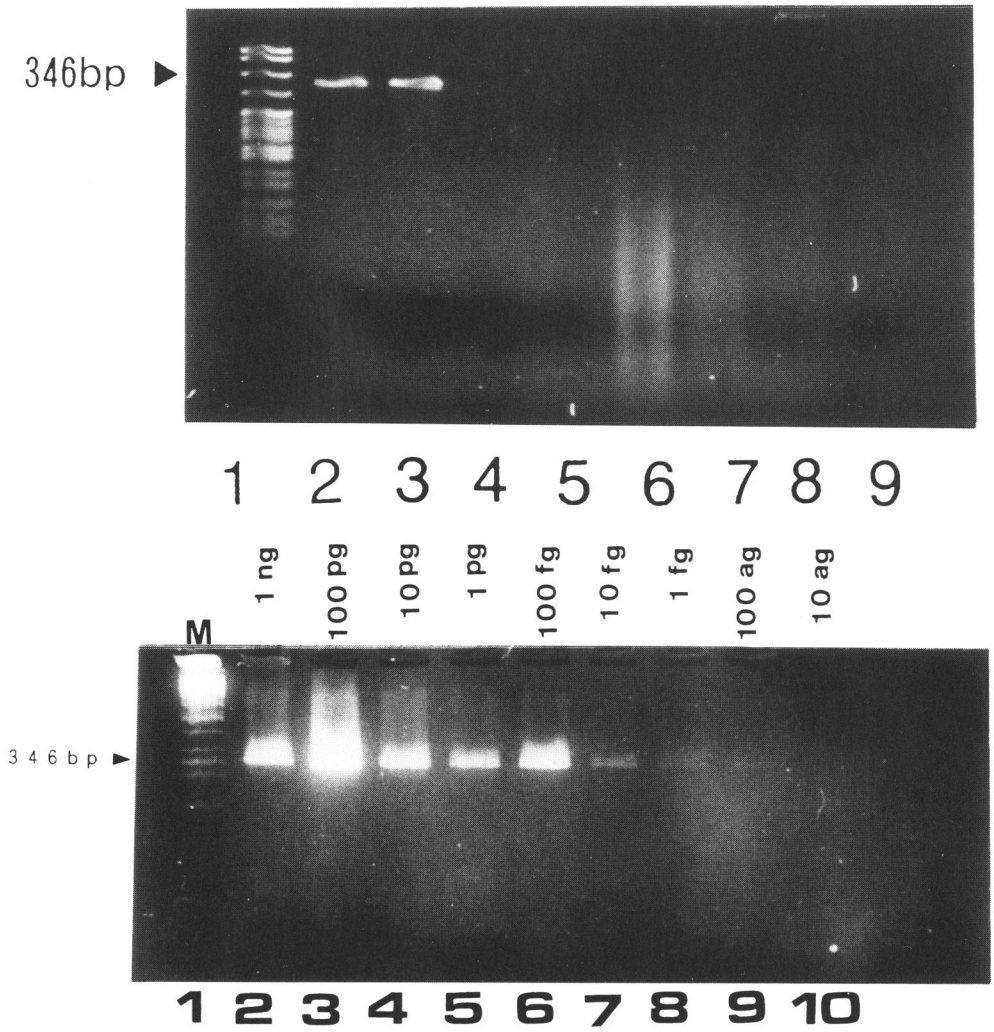

Fig. 3 a. Specificity of amplification using designed primer pairs
One microgram of DNA of HCMV (AD169 and Davis strains), HSV-1 F strain, HSV-2 G strain, VZV Oka strain, Raji cell infected with EBV, HHV-6 Hashimoto strain and MRC-5 cells were amplified 30 cycles by PCR using the PIE-1 and PIE-2 primers (lane 2: HCMV AD169, lane 3: HCMV Davis, lane 4: HSV-1, lane 5: HSV-2, lane 6: VZV, lane 7: Raji infected with EBV, lane 8: HHV-6, lane 9: MRC-5). Amplified products were analyzed by agarose gel electrophoresis as described in Fig. 2. An arrowhead indicates the amplified DNA fragment of 346 bp. Lane 1: Size marker, HincII fragments of Φ X174 DNA

b. Sensitivity of PCR
A serial dilution of the pAD346 plasmid carrying 346 bp PCR product of HCMV IE-1 DNA was mixed with 1 μg of MRC-5 cell DNA and was amplified 30 cycles using the PIE-1 and PIE-2 primers. Amplified products were analyzed by agarose gel electrophoresis. The amounts of the input plasmid DNA are shown on the top of the gel. The arrowhead indicates the amplified 346 bp DNA. Lane 1: Size marker HincII fragment of Φ X174 DNA

which contains the 346 bp PCR product of HCMV IE-1 gene (Fig. 1), was serially diluted and subjected to 30 cycles of amplification (Fig. 3-b). As little as 1 fg plasmid DNA, equivalent to 0.079 pg HCMV DNA, was amplified and detected (Fig. 3-b, lane 8).

Detections of viral mRNA in clinical specimens.

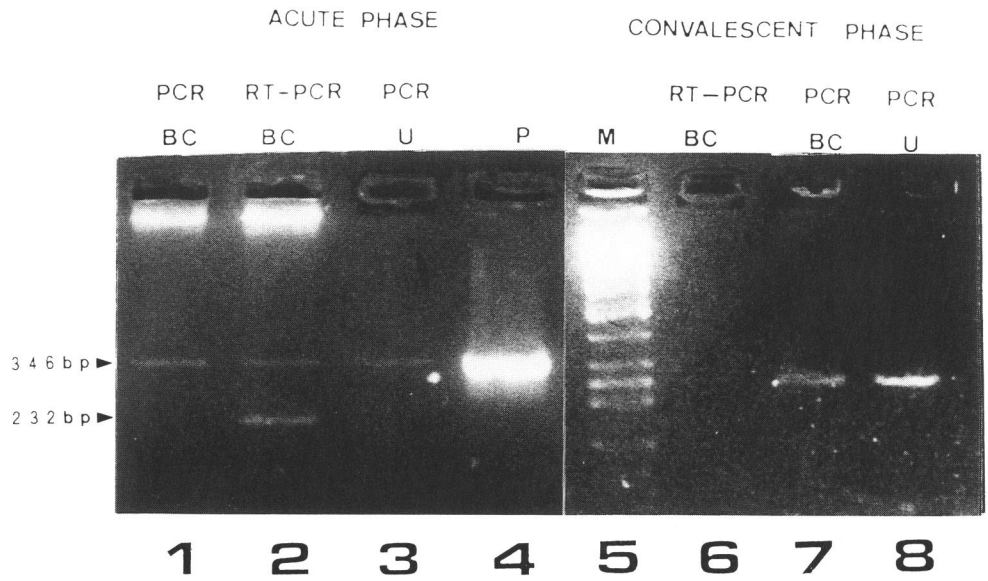

Fig. 4 Detection of viral DNA and mRNA in clinical specimens by RT-PCR.
Blood and urine specimens from a hepatitis patient (Case 1 in Table 1) were analyzed by RT-PCR or PCR in the acute and convalescent phases of clinical symptoms. DNA from buffy coat (BC, lane 1 and 7) or urine (U, lanes 3 and 8) and total RNA from buffy coat (BC, lanes 2 and 6) were amplified by PCR or RT-PCR, respectively, using the PIE-1 and PIE-2 primers. Lane 4: positive control HCMV AD169 DNA (P), Lane 5: Size marker (M), HincII fragments of Φ X174

For clinical applications of our PCR and RT-PCR methods, urine and blood specimens were obtained from 3 patients isolated HCMV from urine; their clinical and laboratory data were summarized in Table 1. During the acute phase, HCMV was isolated from their urine. In this case anti-HCMV IgM antibody (raised against HCMV early and late antigen) was detected in all patients. RT-PCR assay indicated HCMV IE-1-specific mRNA in buffy coats of 1 patient (case 1). HCMV IE-1-specific DNA was detected in buffy coats of all patients by PCR. During the convalescent phase, HCMV IE-1 DNA remained detectable but the IE-1-specific mRNA was not found in any cases.

RT-PCR products in a hepatitis patient (case 1) are shown in Fig. 4. The fragment of 232 bp which represents the IE-1-specific mRNA was amplified by RT-PCR only in the acute phase using buffy coat (compare lanes 2 and 6 in Fig. 4). The DNA fragment of 346 bp was amplified by PCR in either phase of illness using buffy coat as well as urine (Fig. 4 lanes 1, 3, 7 and 8). At the time HCMV IE-1-specific mRNA was detected in case 1, his liver function was bad and deteriorating, judging from GOT and GPT (data not shown). When the mRNA was not detected in case 1, his liver function was also improving. Thus, the IE-1-specific mRNA detected by RT-PCR in the buffy coats of this patient showed a relation to his clinical symptoms.

Discussion

We described a method for detection of HCMV IE-1-specific mRNA by RT-PCR. Using the originally designed primers, RT-PCR enabled us to detect HCMV IE-1-specific mRNA in HCMV-infected tissue culture cells and in blood specimens from patients suspected of HCMV infection. Detection of HCMV sequences by PCR is sensitive and rapid, with virus production and conventional blot hybridization methods. To estimate false positive signals by PCR, we confirmed amplified products by filter hybridization.

HCMV DNA has been shown by PCR assay to be present in various tissues: urine of infants with congenital HCMV infection and peripheral blood of organ transplanted patients (5, 6, 7). These report-

ed the existence of HCMV DNA, but did not show the expression of viral genes. There have been few reports of RT-PCR assay for HCMV (14, 15), but this test may prove to be very useful, as it indicates not only the presence of this potentially deadly virus, but also whether it has begun propagating. Therefore we attempted to verify the activation of the viral genome by searching for viral mRNA using RT-PCR. We chose IE-1-specific mRNA because the expression of the IE-1 gene is a necessary part of HCMV propagation (16). We detected IE-1-specific mRNA in HCMV-infected tissue culture cells by RT-PCR. We applied our PCR and RT-PCR methods to detect HCMV DNA and mRNA in some clinical specimens. All of the 3 patients examined were positive for HCMV IE-1 DNA, but the IE-1 specific mRNA was only detected in one patient in the acute phase. In acute phase of cases 2 and 3, no IE-1-specific mRNA was detected, although the virus was isolated. This might be explained as follows. First, IE-1 mRNA was probably present, but at a level beneath the limit of detection by RT-PCR. This could be resolved using the nested PCR method (17). Second, sources of samples used for virus isolation and RT-PCR were different (urine and buffy coat). Finally, samples for virus isolation and for the RT-PCR assay were prepared with a time lag about 1 week. In the convalescent phase of case 2, IgM antibody was negative, in spite of positive virus isolation.

HCMV shedding can happen in urine of healthy children, irrespective of the presence of IgM antibody (18). In cases 1 and 3, this may be what had occurred, since the HCMV persistent infection was confirmed in the convalescent phase. The HCMV genome has been announced to be detected in peripheral blood from some healthy donors (19, 20, 21). In our preliminary screening of over one hundred healthy blood donors, HCMV IE-1 specific DNA was detected in some samples. However, HCMV IE-1 specific mRNA was never detected in any of HCMV DNA-positive samples by RT-PCR (unpublished data).

Blood transfusion allows some HCMV diseases to disseminate; many are triggered by the reactivation of HCMV (22). RT-PCR may be applicable for examination for reactivated HCMV which persists in blood or organs. For further assessment of the clinical significance, we are currently investigating different species of HCMV mRNA in patients at various stages of HCMV infection.

Conclusion

We have established a method for detection of HCMV IE-specific mRNA by RT-PCR. RT-PCR is readily applicable for rapid detection of HCMV mRNA and should prove useful for diagnosis of active primary or recurrent HCMV infections.

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RT-PCR法によるヒトサイトメガロウイルス前初期遺伝子mRNAの検出

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抄 録

我々は，逆転写酵素を用いたPolymerase Chain Reaction（RT-PCR）法によるヒトサイトメガロウイルス（HCMV）前初期遺伝子（IE-gene）mRNAの検出法を確立した．IE-1 geneに特異的なprimerを設定し，これを用いたRT-PCR法により，HCMV AD169株のIE-1 mRNAに対応する232塩基対の長さのDNA断片を増幅できた．またこのprimerは，HCMVに特異的であり他のヘルペスウイルスDNAやヒトDNAを増幅しなかった．さらにHCMV感染培養細胞，およびHCMVが尿より分離された3人の患者中1人の血液検体においてHCMVのIE-1 mRNAを検出した．RT-PCR法はHCMV mRNAの迅速で簡便な検出に，HCMV感染の診断，把握に有用であると考えられる．