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Diagnosis and monitoring of acute cytomegalovirus infection in peripheral blood of transplant recipients by nested reverse transcriptase polymerase chain reaction (RT-PCR)

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Abstract The aim of this novel diagnostic approach is to monitor cytomegalovirus (CMV) infection in immunocompromised transplant recipients using early, sensitive, and specific predictors before and during antiviral therapy. The peripheral blood cells of 20 patients after transplantation (9 liver, 7 kidney, 4 simultaneous kidney-pancreas) were studied for an early diagnosis of acute infection. The mRNA and DNA of human CMV immediate-early antigen (IEA) were detected by nested-polymerase chain reaction (PCR) assay. Results of nested PCR were compared with the immunological detection of antigen pp65 and serological diagnosis of CMV infection. All data were correlated with clinical symptoms like leukopenia, thrombopenia, pneumonia, and allograft-rejection reaction. Of 20 transplant recipients, 12 were infected by CMV, and 9 suffered from a CMV-related disease. CMV mRNA were detected simultaneously with antigen pp65 and CMV DNA in all patients with symptomatic infection. Additionally, CMV mRNA was found over a longer period after ganciclovir treatment of infected recipients. Nested reverse transcriptase (RT)-PCR for CMV-IEA mRNA allows a sensitive and specific diagnosis of an acute CMV infection. CMV mRNA was found to be a good marker of acute viremia and could be a useful tool for CMV monitoring over the whole period of disease management, even during antiviral therapy.

Key words Acute CMV infection · CMV mRNA · Nested RT-PCR · Transplanted patients

Introduction

An early and sensitive laboratory diagnosis of acute human cytomegalovirus (CMV) infection is of enormous importance in monitoring transplanted patients. CMV is associated with allograft rejection symptoms and severe morbidity and mortality in immunosuppressed transplant recipients. Main sources of postoperative CMV infection include transfusion of blood or blood components, the graft tissue, and infection of the recipient before transplantation [1, 11, 15]. A rapid diagnosis of acute viremia is essential to overcome the rejection of the transplanted organ by application of an effective antiviral therapy and to avoid overtreatment by immunosuppressive drugs [1, 3, 9, 10, 12, 15, 16, 19–21].

The diagnosis of CMV consists of three different states: (1) no infection (absence of CMV DNA, CMV RNA, antigens, and antibodies), (2) viremia (presence of CMV DNA, CMV RNA, antibodies, and antigens), and (3) latent infection by CMV (presence of CMV DNA and antibodies, absence of CMV RNA and antigens). DNA amplification techniques provide the potential to detect low copy numbers of viral nucleic acids. Nearly all reports described the amplification of CMV DNA for detection of acute infection, but several have shown that the presence of viral DNA does not correlate with symptomatic infection [1, 2, 7, 11, 15]. Prob-

lems arise concerning positive tests as the infection marker. Some 50%–60% of all healthy blood donors are seropositive and therefore able to transmit the virus [2, 14]. Very sensitive polymerase chain reaction (PCR) assays allow the detection of CMV DNA during a latent infection [3, 7, 14, 15]. Different clinical specimens (serum, urine, whole blood) were analyzed for discrimination between latent and acute infection by CMV [7, 9, 11]. The only reliable diagnosis of acute CMV infection involves the detection of viral RNA or viral antigens like pp65 [1, 5, 14, 20].

The aim of this study was the evaluation of CMV immediate-early antigen (IEA) mRNA as a relevant marker for discrimination between a latent CMV infection and viremia. We established a nested reverse transcriptase (RT)-PCR for CMV-IEA-mRNA with specific primers. Results were correlated with the detection of antigen pp65 [11], nested-DNA PCR [3, 16, 17, 19], and clinical symptoms of a CMV-related disease.

Materials and methods

Clinical specimen

A total of 148 blood samples from 20 transplant recipients (9 hepatic, 7 renal, and 4 simultaneous renal-pancreatic) was studied for CMV infection. Samples were obtained by venipuncture and processed independently for different parameters. Sera were tested for CMV-specific antibodies, and leukocytes were examined for the presence of CMV-antigen pp65 and viral nucleic acids. Blood samples were obtained weekly during the inpatient period and, for most patients, every 2 weeks during a 100-day period. After these 100 days, patients were analyzed occasionally at outpatient visits. CMV infection was diagnosed by CMV antigen pp65, positive DNA-PCR, and seroconversion. Additionally, CMV mRNA was estimated simultaneously by nested RT-PCR. Clinical syndromes of acute CMV infection were defined as fever, arthralgia, leucopenia, thrombocytopenia, pneumonia, and allograft-rejection symptoms.

Nucleic acid preparation

Leukocytes were isolated from 2.8 ml of peripheral blood after lysis of erythrocytes. After addition of 3-fold volume of lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylene diamine tetraacetic acid (EDTA), the blood samples were incubated for 15 min on ice. Cells were sedimented by centrifugation (600 × *g*, 10 min) and washed twice with 2.5 ml of lysis buffer. The total RNA was extracted from sedimented cells according to Chomczynski and Sacchi [5]. Genomic DNA was isolated by DNAzol method [4].

Amplification of CMV-IEA sequences

Detection of CMV-IEA mRNA was performed by a single-tube RT-PCR using specific primers and rTth DNA polymerase (Perkin Elmer, Norwalk, USA). Reverse transpription of mRNA and subsequent amplification of the generated IEA-cDNA were performed in the same reaction vessel [13]. The 1-µg samples of isolated total RNA were reverse transcribed in 20 µl of RT mixture containing 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 0.2 mM of each dNTP, 1.0 mM $MnCl_2$, 100 pmol of downstream primer CP2, and 2.5 units rTth DNA polymerase. The RT reaction was performed by the "hot start" technique (60 °C) [8] and followed by incubation for 3 min at 72 °C, 10 min at 52 °C, and 3 min at 72 °C. Then the tubes were cooled on ice, and 80 µl of a PCR mix were added, containing a final concentration of 10 mM Tris-HCl (pH 8.3), 5% glycerol (v/v), 100 mM KCl, 0.75 mM ethylene *bis*-(oxyethylenenitrilo)tetraacetic acid (EGTA), 2.5 mM $MgCl_2$, and 100 pmol of upstream primer CP3. PCR was performed by the "hot start" technique and amplification over 40 cycles (94 °C for 10 s, 52 °C for 10 s, 72 °C for 30 s; initial denaturation 94 °C for 5 min). CMV-DNA was amplified in a 50 µl PCR reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatine, 2 mM each of dATP, dCTP, dGTP, and dUTP, 50 pmol of each outer primer (CP2, CP3), and 1.5 units AmpliTaq DNA polymerase (Perkin Elmer) by the above mentioned amplification profile. For detection of low copy numbers of analysed gene transcripts, 5-µl aliquotes of RT-PCR or CMV-DNA-PCR were amplified in a 50 µl PCR using a second inner primer pair (CI1, CI2). This nested PCR was performed by the "hot start" technique and amplification over 40 cycles (94 °C for 10 s, 58 °C for 10 s, 72 °C for 30 s; initial denaturation 94 °C for 5 min). The sequences of primers and probe are listed below and were synthesised by custom order (MWG BIOTECH, Ebersberg, Germany). The sequences were based on the mRNA and DNA sequences of IEA of human CMV [6]: CP2: CAG CAC CAT CCT CCT CCT CCT CCT CTG G; CP3: CCA AGC GGC CTC TGA TAA CCA AGC C; CI1: AGA GTC TGC TCT CCT AGT GTG; CI2: AGA CAC TGG CTC AGA CTT GAC. A 10-µl PCR sample was run on 2% (w/v) Qualex Gold agarose (AGS, Heidelberg, Germany) gel in 1 × TAE buffer supplemented with ethidium bromide (4 µl/100 ml buffer). Clearly visible bands with 215 bp in length were considered positive PCR results.

Determination of virus-specific antibodies and CMV antigen pp65

All recipients were screened before and after transplantation for virus-specific antibodies. The donor's serum samples were analyzed at the moment of transplantation. CMV IgM and IgG were measured by Cobas Core ELISA kit and analyzer (Roche, Basel, Switzerland). CMV antigen pp65 staining was performed by the cytospin method with specific antibodies C-10, C-11 (Biotest, Dreieich, Germany), as described by Van der Bij et al. [21].

Ganciclovir treatment

Ganciclovir was applied intravenously at a standard dosage of 10 mg per kg per day for 14 days to five recipients with mismatched CMV status (donor + /recipient–) as preventive antiviral therapy immediately after transplantation and to one patient (donor–/recipient–) after diagnosis of primary infection over a period of 1 month. In addition, 4/5 antibody-negative recipients with a CMV-positive organ were treated with a second course of ganciclovir because of a primary symptomatic infection. Two patients with a recurrent infection (donor + /recipient + ; donor–/recipient +) underwent an antiviral therapy for to 1 month to manage the infection successfully.

Statistical analysis

Diagnostic results were compared with the occurrence of clinical symptoms of CMV-related disease according to Evans et al. [9]. Sensitivity was defined as the proportion of disease episodes per patient that tested positive. Specificity was defined as the proportion of no-disease episodes per patient that tested negative. Positive predictive value was defined as the proportion of "test positive" episodes per patient suffering from disease. Negative predictive value was defined as the proportion of "test negative" episodes per patient who was disease-free.

Table 1 Incidence of cytomegalovirus (CMV) infection and CMV-related disease in relation to donor/recipient status in 20 transplantation patients (9 liver, 7 kidney, 4 simultaneous kidney-pancreas). Status refers to presence (+) or absence (−) of CMV immunoglobulin (Ig)G

CMV antibody status	No. with CMV infection (%)	No. of infected patients with CMV-related disease (%)
Donor +, recipient +	2/5 (40)	1/2 (50)
Donor +, recipient −	4/5 (80)	3/4 (75)
Donor −, recipient +	5/7 (71)	4/5 (80)
Donor −, recipient −	1/3 (33)	1/1 (100)
Total	12/20 (60)	9/12 (75)

Results

In total, 148 blood samples of 20 transplant recipients were analyzed for CMV infection. CMV infection, defined by the detection of CMV nucleic acids by nested PCR, occurrence of pp65, and seroconversion, was determined in 12 of 20 (60%) transplant recipients. Detectable CMV infection was found in 6/7 (86%) recipients with renal transplants (KTx), in 5/9 (56%) patients with hepatic transplants (LTx), and in 1/4 (25%) recipients after simultaneous kidney-pancreas transplantation (KPTx).

Nine (75%) of the CMV-infected recipients showed clinical symptomes of acute infection like leukopenia or thrombopenia and underwent antiviral therapy. In one serious case, the recipient developed additionally a CMV-mediated pneumonia and finally an allograft-rejection syndrome which required the removal of the transplanted organ (see Fig. 1, patient 3). Three of these 9 patients were not treated with ganciclovir because they had only mild clinical complications. For 3 patients with diagnosed CMV infection, no clinical symptoms were observed. These recipients (3/3) were positive for CMV antibodies before transplantation and received an organ from a CMV IgG-negative donor. In general, risk of disease was higher in patients with renal transplants (4/7) than in patients after liver or simultaneous kidney-pancreas transplantations (4/9 LTx, 1/4 KPTx).

The results of pretransplant serology in correlation with post-transplant CMV infection and disease are shown in Table 1. Recipients with a mismatched CMV antibody status [9/12 (75%)] showed a higher incidence of subsequent CMV infections in comparison with recipients with identical antibody status [3/8 (38%)]. Symptomatic CMV infection occurred in 7/9 (78%) recipients with mismatched serological status. 2/3 recipients with identical CMV antibody status developed a CMV-related disease.

The postoperative period was divided into disease-free and disease-related episodes [9]. The presence of CMV-IEA DNA or the corresponding mRNA was correlated with the occurrence of antigen pp65 and clinical symptoms (Table 2). Eight recipients (4 LTx, 1 KTx, 3 KPTx) had no detectable CMV infection. Pretransplant serology of the donor/recipient was identical in 5/8 (63%) of these patients. Two of 8 seropositive patients received an organ from a seronegative donor. One of seronegative recipient received a transplant from a seropositive donor. The risk of primary infection was reduced in this case by preventive antiviral therapy. No CMV DNA or mRNA was found in all samples taken from these patients [47 blood samples over a median 36 days (range 0–132 days)] by nested PCR.

Table 2 Nested polymerase chair reaction (PCR) of DNA and mRNA and pp65 test in relation to CMV-related disease in 12 infected transplant recipients up to 200 days post-operation

	Nested DNA-PCR		Nested RT-PCR		pp65 test	
	+	−	+	−	+	−
CMV disease-free episodes	10	27	12	25	0	17
CMV-related disease episodes	23	5	20	8	11	17

CMV mRNA-positive results were achieved at a median 55 days (range 20–164 days) and CMV DNA-positive values at a median of 58 days (range 17–266 days) after transplantation. The duration of the CMV-related disease was not associated with the presence of both viral nucleic acids (DNA and mRNA). Typical case studies of patients with CMV-related disease are shown in Fig. 1. In contrast to antigen pp65, the nested PCR for CMV mRNA and DNA were more suitable for long-term monitoring of CMV infection because positive signals were achieved over a longer period. In one case, the CMV mRNA was found during 70 days after the last clinical symptoms of CMV-related disease (Fig. 1, patient 2). In 3/12 patients with asymptomatic infection, CMV DNA and mRNA were detected simultaneously (in one recipient over a period of 20 consecutive days).

PCR assays for CMV nucleic acids gained higher values of sensitivity and positive predictive value than the pp65 test (Table 3). Antigen pp65 was important to ex-

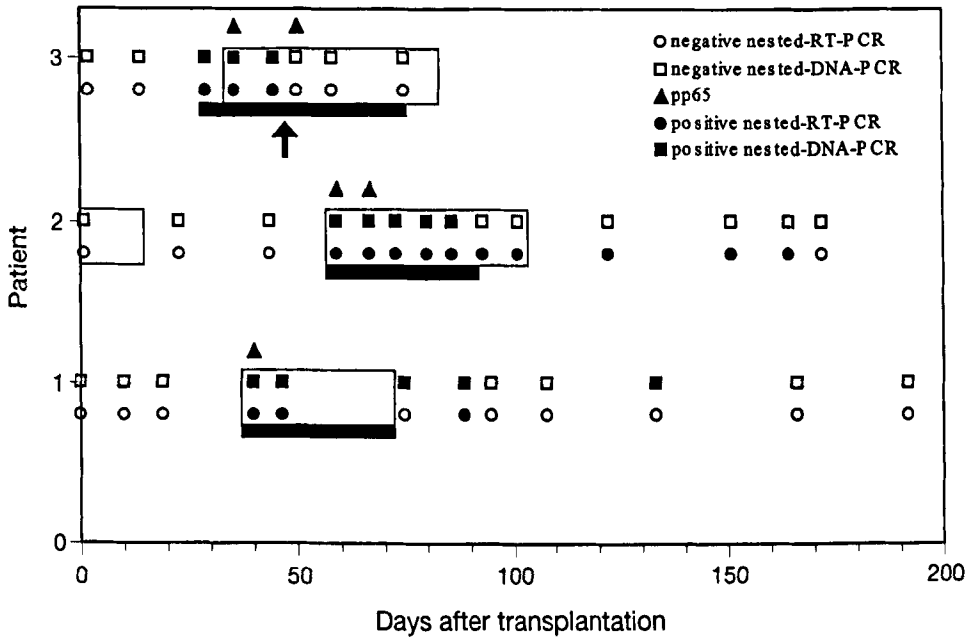

Fig. 1 Molecular diagnostics of three transplant recipients with symptomatic CMV infection. Patient 1: matched CMV status (donor–, recipient–) with primary infection of unknown origin; nested PCR for CMV mRNA and DNA and pp65 test were initially positive on day 40, start of antiviral therapy (*box*), remained PCR positive until day 89. Patient 2: mismatched CMV status (donor +, recipient–) with primary infection by transplanted organ, received preventive ganciclovir treatment until day 14; pp65 and nested PCR for viral sequences were initially positive on day 58, start of antiviral therapy, disappearance of pp65 after day 67, remained positive for CMV DNA until day 83 and for CMV mRNA until day 164. Patient 3: identical CMV status (donor +, recipient +) with reactivation of CMV infection; nested PCR for CMV mRNA and DNA were initially positive on day 29 but pp65 test was negative until day 36, patient developed a CMV pneumonia and finally the transplant was removed on day 44 (*arrow*). Duration of disease is shown as *black bar*

clude a CMV-related disease (specificity and negative predictive value 100%). The low values of sensitivity and positive prediction were based on the rapid disappearence of this antigen after starting viral therapy even in the period of symptomatic CMV infection.

Table 3 Sensitivity, specificity, and predictive values of nested PCR of DNA and mRNA and pp65 test in relation to CMV-related disease

	Nested DNA-PCR	Nested RT-PCR	pp65 test
Sensitivity	82%	71%	39%
Specificity	73%	68%	100%
Positive predictive value	84%	84%	42%
Negative predictive value	54%	54%	100%

The occurrence of pp65 antigen is considered the classic marker of current symptomatic CMV infection and signals the need for an antiviral therapy. In one case of a CMV-related disease, positive results were achieved 1 week earlier by nested RT-PCR than by the antigen test (Fig. 1, patient 3). This recipient showed many of the characteristic features of severe CMV infection like leukopenia, thrombopenia, fever, and CMV pneumonia, which finally demanded a removal of the transplanted organ.

Discussion

CMV is a major cause of morbidity and mortality in immunocompromised transplantation patients [1, 3, 7, 9–11, 15–17, 19–22]. CMV viremia is a reliable marker for systemic infection and has a high predictive value for the occurrence of clinical symptoms of CMV related disease. Viremia is the phase of intensive replication of viral genomes, accompanied by increasing amounts of viral RNA. Therefore, CMV mRNA is a marker of acute viremia and for monitoring of CMV infection.

Many efforts have been made to discriminate between latent CMV infection and acute viremia. The viral antigen pp65 is considered to be a key parameter. Antigen-positive cells were detectable before any other diagnostic result was obtained [13]. In practice, the estimation of antigenemia by the pp65 test determines the start of antiviral therapy by ganciclovir. However, this treatment makes monitoring of the CMV infection by the pp65 antigen impossible because in the following days all pp65-positive cells are eradicated from the peripheral blood [1, 10, 20]. Monitoring of CMV infection

by serological testing is limited in immunocompromised transplant patients [21, 22]. The only remaining diagnostic possibility is the detection of viral nucleic acids by DNA amplification methods. Detection of CMV DNA in peripheral blood cells does not imply an acute infection [2, 7, 9, 11]. Because CMV is a cell associated virus, the presence of CMV DNA in serum samples indicates an acute infection [9, 11, 16]. Another approach is the quantification of CMV genomes and the following correlation to infection status [9, 16, 19]. A direct attempt to detect CMV viremia is the prove of viral RNA in peripheral blood leukocytes [14, 15].

We developed a nested RT-PCR for CMV-IEA mRNA. Nested PCR is a very sensitive and specific method based upon the second amplification step and the selective properties of the second inner primer pair [3, 16, 17, 19]. Diagnostic PCR assays based upon the immediate-early gene sequences have been used frequently for the detection of CMV [2, 3, 7, 12, 15, 19]. Results of the nested-RT-PCR were correlated with the simultaneous presence of CMV DNA and the antigen pp65 test.

In our study, the incidence of CMV infection and related disease after transplantation (KTx, 86% vs 57%; LTx, 56% vs 44%; KPTx, 25% vs 25%) is consistent with other reports [1, 7, 9, 10, 17]. Mismatched CMV antibody status (donor + /recipient– or donor–/recipient +) is a risk factor for CMV infection in the posttransplantation period. In contrast, recipients with identical pretransplant serology have a distinctly lower incidence of CMV infection (36% vs 75%). Similar results were reported [1, 9, 10, 17, 19, 20].

The sensitivity, specificity, and positive predictive value of nested PCR were comparable for viral DNA and mRNA but different from the detection of viral antigen pp65. One reason for the low negative predictive values of our nested RT-PCR (54%) was the high sensitivity. A few copies of CMV sequences could be measured even after the last onset of disease. In contrast to the pp65 test and nested DNA-PCR, we could detect IEA mRNA over a period of 70 days after the last disease episode and applied ganciclovir treatment.

The nested RT-PCR could give more insights into the behavior of CMV in peripheral blood cells. Especially the discrimination between latent and acute CMV infection and the correspondance of determination of CMV mRNA with the period of symptomatic CMV infection are targets of nested RT-PCR in clinical diagnostics.

Although the nested RT-PCR for CMV-IEA mRNA is a very sensitive technique for the detection of acute CMV infection, it is not the only predictive marker of acute viremia. However, in addition to current applied diagnostic methods, it may yield valuable information, even during antiviral therapy of symptomatic CMV infection. Further studies are under way to confirm the initial results for monitoring the efficacy of antiviral therapy.

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