IN SITU RT-PCR CAN DISTINGUISH BETWEEN PRODUCTIVE AND LATENT CYTOMEGALOVIRUS INFECTION IN THE BLOOD CELLS OF BONE MARROW TRANSPLANT RECIPIENTS

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Summary. – Thirty-four peripheral blood leukocyte samples from bone marrow transplant (BMT) recipients were examined for Human cytomegalovirus (HCMV) phosphoprotein 65 (pp65), DNA and late transcripts. Twenty seven samples were positive for pp65 in the cytoplasm by immunofluorescent assay (IFA). Viral DNA was confirmed in 26 samples by nested PCR (nPCR). Using *in situ* RT-PCR, viral late transcripts were found in 19 samples, positive also by IFA and nPCR; these samples were considered indicative of productive viral infection. Five samples, positive by nPCR but negative by IFA and *in situ* RT-PCR, were considered to represent latent viral infection. In 8 samples, positive by IFA and nPCR but negative by *in situ* RT-PCR, apparently phagocytosis of viral particles took place.

Key words: Human cytomegalovirus; in situ RT-PCR; pp65 antigen; viral DNA; viral transcripts; bone marrow transplant patients

Introduction

HCMV (the species *Human cytomegalovirus*, the genus *Cytomegalovirus*) causes asymptomatic infection in immunocompetent persons (Alford and Britt, 1996). Following primary infection, the virus establishes lifelong latent infection. The latency is controlled by a functioning immune system, but its dysfunction leads to increased viral replication (Hengel *et al.,* 1998; Streblow and Nelson, 2003). Reactivation of latent or primary HCMV infection is an important cause of morbidity and mortality in transplant recipients and patients infected with HIV (Alford and Britt, 1996). In bone marrow transplant (BMT) recipients, the most threatening diseases are HCMV pneumonia and gastroenteritis (Hengel *et al.,* 1998; de Jong *et al.,* 1998; Landolfo *et al.,* 2003).

Development of a diagnostic test would correctly indicate the risk of HCMV disease is very important in the success of preemptive or prophylactic therapy. Rapid, sensitive, specific and reliable diagnostic assays for HCMV are essential for achieving these goals (Boeckh and Boivin, 1998).

The methods assessing the virus, viral antigen (pp65) and viral DNA in the blood are recommended for the patients suffering from cytomegalovirus disease (Preiser *et al.,* 2001; Razonable *et al.,* 2002; Schafer *et al.,* 1997).

The assay of pp65 in the blood allows for the monitoring of dynamic changes in virus load (Gerna *et al.,* 1992). HCMV antigenemia is not detectable in latent infection and is a marker for productive viral infection (Amorim, 2001). The method is based on direct detection of HCMV pp65 in the nucleus of leukocytes (Perez *et al.,* 1994). However, according to our own and other observations, pp65 is

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Abbreviations: BMT = bone marrow transplant; gB = glycoprotein B; HCMV = Human cytomegalovirus; IFA = immunofluorescent assay; NASBA = nucleic acid sequence-based amplification; nPCR = nested polymerase chain reaction; pp65 = phosphoprotein 65

Table 1. Primers used in nested PCR and *in situ* RT-PCR

Primers		Sequence
Outer set	HCMV(gB)ex	5'-GTCGACGGTGGAGATACTGCTGAGG-3'
	HCMV(gB)ex	5'-GAGGACAACGAAATCCTGTTGGGCA-3'
Inner set	HCMV(gB)in	5'-ACCACCGCACTGAGGAATGTCAG-3'
	HCMV(gB)in	5'-TCAATCATGCGTTTGAGGAGGTA-3'

frequently found also in the cytoplasm; this may be a result of phagocytosis or accumulation of virions in replicative cycle (Kas-Deelen *et al.,* 2001). On the other hand, PCR, by detecting viral DNA, detect latent (non-replicating) as well as replicating viral genome (Taylor-Wiedeman *et al.,* 1991).

To avoid these problems, the recently introduced semiquantitative or quantitative methods, such as real-time PCR could be used (Ikewaki *et al.,* 2005). These methods have more advantages in comparison to qualitative PCR or antigenemia assays (Caliendo *et al.,* 2001; Yakushiji *et al.,* 2002). They can determine the viral load and therefore are more reliable, especially in immunocompromised patients. Another good indicator of viral replication is viral mRNA in the cell. For example, the recently introduced nucleic acid sequence-based amplification (NASBA) allows for specific amplification of unspliced mRNA over a DNA background (Deiman *et al.,* 2002; Gerna *et al.,* 2000).

In this report, we describe another method, an *in situ* RT-PCR for late gB gene transcripts that can be helpful in discriminating between latent (the transcripts absent) and productive (the transcripts present) HCMV infection in the blood leukocytes.

Materials and Methods

Leukocyte specimens were obtained from 26 BMT recipients in different stage of treatment (in average 7.3 ± 6.2 months after transplantation). The leukocytes were prepared by sedimentation of 3–5 ml EDTA-treated blood samples in 6% dextran solution (The *et al*., 1995). Aliquots of 2×10^5 cells per slide were used to prepare cytospins. The slides were fixed in 5% (for pp65 assay) or 10% (for *in situ* RT-PCR) buffered formalin for 15 mins and 4 hrs, respectively. About 5×10^6 leukocytes were used for isolation of DNA using the Genomic DNA Prep Plus Kit (A & A Biotechnology, Poland).

Quantitative IFA of pp65 was performed according to Gerna *et al*. (1992). A MAb NCL-HCMV pp65 (Novocastra, United Kingdom) was used.

Viral DNA assay by nPCR. Two primer pairs (Table 1) were used to amplify viral glycoprotein B (gB) gene in a thermocycler (Biometra, Germany) according to Mitchell *et al*. (1994). The reaction mixture consisted of 10 mmol/l Tris-HCl pH 8.3; 50 mmol/l KCl; 1.5 mmol/l $MgCl_2$; 1U of Taq polymerase DyNAzyme™ (Finnezymes, Finland); 200 µmol/l, dNTPs; 100 ng of each primer and 20 ng of DNA isolated from leukocytes. In the reaction, negative and positive controls were included. A check for the presence of Taq DNA polymerase inhibitors was performed. The nPCR products were electrophoresed in 2% agarose gel with ethidium bromide.

Assay of viral transcripts by in situ RT-PCR was done in a thermocycler (Perkin Elmer, USA) according to Lewis (1996) and Nuovo (1996). The samples were treated with pepsin (2 mg/ml) for 30 mins at room temperature, inactivated in 100% ethanol and digested with DNase (1 U/µl) at 37°C for 12 hrs. The reaction was carried out on a slide in a volume of 25 µl. The reaction mixture contained 1x buffer EZ (Applied Biosystem, USA), 4 mmol/l $MnCl_2$, 300 µmol/l dNTPs, 15 µmol/l DIG-dUTP, 1 µmol/l of each primer (Table 1), 0.2U/µl rTth DNA polymerase (Applied Biosystem, USA) and 0.5 U/µl RNasin (Applied Biosystem, USA). The *in situ* RT-PCR with the above primers was specific for late gB gene transcripts. A negative control consisting of the reaction without primers was included. The reaction conditions were as follows: 65°C/30 mins (initial incubation), 94°C/3 mins (preliminary denaturation), 20 cycles of 60°C/1.5 min and 94°C/45 secs. The hapten incorporated into the amplified product was detected using an anti-DIG antibody conjugated to alkaline phosphate. The product was visualized by NBT and BCIP according to the manufacturer's instructions (Boehringer Mannheim, Germany).

Table 2. Presence of pp65 HCMV DNA, and gB gene transcripts in 34 blood leukocyte samples from BMT recipients

No. of samples	pp65 (IFA)	Viral DNA (nPCR)	gB gene transcripts (*in situ* RT-PCR)	Interpretation of results (No. of samples)
14	+	+	+	Productive infection (19)
5	+	Inh	+	
7	+	+	–	Phagocytosis ? (8)
1	+	Inh	–	
5	–	+	–	Latent infection (5)
2	–	–	–	No infection (2)

Inh = inhibitors of Taq DNA polymerase present.

Results and Discussion

In this study, 34 samples of human leukocytes were examined for pp65, viral DNA and viral gB gene transcripts. Samples obtained from seronegative persons were used as negative controls. The results are shown in Table 2.

Twenty seven pp65-positive samples, in which the antigen was present only in cytoplasm, and 7 pp65-negative samples were recorded. Using the nPCR, viral DNA was detected in 26 samples; 2 samples were negative and the remaining 6 samples gave unreadable results due to the presence of DNA polymerase inhibitors. Using the *in situ* RT-PCR, 19 samples were found positive for late viral transcripts of gB gene, which confirmed productive infection. Fig. 1 shows the result of the *in situ* RT-PCR, in which the transcripts were found in the cytoplasm.

In 8 samples, in which the *in situ* RT-PCR results were negative, the presence of pp65 in the cytoplasm could probably be due to phagocytosis. Among pp65-negative samples, latent HCMV infection was detected in 5 samples on the basis of viral DNA presence and absence of viral late gB gene transcripts.

In immunocompromised patients, especially after organ transplantation, rapid, sensitive and reliable detection of HCMV infection is necessary. In our study, in order to assess productive HCMV infection, the samples were examined by 3 different diagnostic methods.

Quantitative pp65 assay (IFA) is regarded as a valuable and sensitive tool for diagnosing a HCMV disease, although the interpretation of its results is problematic. It is recommend usually to record only the cells with nuclear fluorescence. However, in our study, such cells were rare, as leukocytes with a positive signal in the cytoplasm occurred more frequently.

We utilized also nPCR, which is useful mainly because of its high sensitivity. However, it cannot discriminate between latent and productive infection. Moreover, this test gives sometimes unreadable results due to the presence of Taq DNA polymerase inhibitors in samples.

Therefore we used the *in situ* RT-PCR detecting late transcripts of viral gB gene to confirm productive viral infection. In general, late transcripts are better markers of productive HCMV infection than immediate-early ones, which can be transcribed also during non-productive infection (Taylor-Wiedeman *et al.,* 1994).

Our studies indicate that the *in situ* RT-PCR could be helpful in unequivocal distinguishing between productive and non-productive (latent) HCMV infection, especially when the results obtained with other methods are discordant. In future, we could consider additional novel approaches. It could be NASBA, which also amplifies viral mRNA (Gerna *et al*., 2000) but is less sophisticated than *in situ* methods and therefore more frequently used. Instead of qualitative PCR we could use quantitative methods, which allow for the determination of viral load. This is especially a good marker for proper prognosis of BMT recipients (Jebbink *et al*., 2003; Nitsche *et al*., 2003).

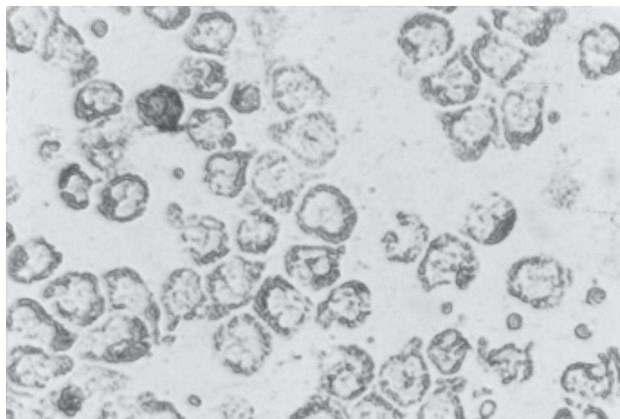

Fig. 1

The presence of HCMV gB gene transcripts in the cytoplasm of blood leukocytes

In situ RT-PCR. Magnification 400x.

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