

In situ detection of DNA and mRNA of human cytomegalovirus to distinguish different forms of viral infection in leukocytes*

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In situ PCR and *in situ* reverse transcription PCR (RT-PCR) were applied to discriminate between latent and productive infection of human cytomegalovirus (HCMV) in leukocytes. We investigated 28 samples, in which viral pp65 antigen was detected only in the cytoplasm of leukocytes. Additionally we assayed 12 specimens lacking pp65 antigen. Using nested PCR (nPCR), viral DNA was detected in 27 samples. In six samples the results of nPCR were unreadable due to the presence of polymerase inhibitors. By application of *in situ* PCR, we were able to confirm the presence of viral DNA in the nucleus and/or cytoplasm. Productive infection was recognized in 20 samples in which transcripts for late viral genes were detected. Among the 20 samples negative by *in situ* RT-PCR, we recognized phagocytosis of viral particles in eight and the latent form of HCMV infection in five.

Keywords: cytomegalovirus, antigenemia pp65, PCR, *in situ* PCR, *in situ* RT-PCR

INTRODUCTION

Human cytomegalovirus (HCMV) infection remains a major problem in immuno-compromised patients, such as bone marrow or solid organ recipients and those suffering from AIDS (Alford & Britt, 1996). Following primary infection, HCMV establishes lifelong latency, which is controlled by a functioning immune system. Immunodeficiency leads to increased levels of HCMV replication and can cause severe, life-threatening diseases (Hengel *et al.*, 1998; Streblow & Nelson, 2003).

Development of a diagnostic test that correctly indicates 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 detection are essential for achieving these goals (Boeckh & Boivin, 1998). Quantitative methods that assess viremia, including

antigenemia assay and PCR techniques (DNAemia), are recommended in patients at risk for HCMV disease for early diagnosis and monitoring of antiviral treatment (Schafer *et al.*, 1997; Preiser *et al.*, 2001; Razonable *et al.*, 2002).

The antigenemia assay is based on direct detection of HCMV phosphoprotein 65 (pp65 antigen), which is found in the nucleus of leukocytes during active infection (Gerna *et al.*, 1992; Perez *et al.*, 1994). Our own and those of others observations reveal that pp65 antigen is also found in the cytoplasm quite frequently, which may be a result of phagocytosis or accumulation of virions in the replicative cycle (Kas-Deelen *et al.*, 2001).

To explain this phenomenon we used additional methods such as nested PCR (nPCR) and *in situ* PCR. Because application of PCR technology for the detection of viral nucleic acid allows one to detect both latent and replicating viral sequences,

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Abbreviations: gB, glycoprotein B; HCMV, human cytomegalovirus; nPCR, nested PCR; pp65, phosphoprotein 65; RT-PCR, reverse transcription PCR.

this approach should be expanded for the detection of HCMV late transcript, which may better reflect productive viral replication (Taylor-Wiedeman *et al.*, 1991; Gozlan *et al.*, 1992).

MATERIALS AND METHODS

Clinical specimens. Forty samples of leukocytes obtained from 32 patients after bone marrow transplantation (BMT) in different stages of treatment (1–24 months after transplantation, average 7.0 ± 6.0) were investigated. Leukocytes were isolated by sedimentation of 3–5 ml EDTA-treated blood samples with 6% dextran solution (The *et al.*, 1995). The upper part was collected after 30 min incubation at 37°C and centrifuged at $600 \times g$ for 15 min. The contaminating erythrocytes were lysed with 0.75% NH_4Cl solution at 4°C for 10 min. Aliquots of 2×10^5 leukocytes per slide were used to prepare cytopins. The slides were fixed in 10% buffered formalin for 4 h (*in situ* PCR and *in situ* RT-PCR) or in 5% buffered formalin for 15 min (antigenemia pp65). The remaining leukocytes (1×10^6 cells) were used to obtain DNA, which was isolated using Genomic DNA Prep Plus (A & A Biotechnology).

pp65 antigenemia. Monoclonal antibodies NCL-HCMV pp65 (Novocastra) were used. The HCMV pp65 antigen was detected according to Gerna *et al.* (1992).

Nested PCR. Two pairs of primers to amplify the gene encoding viral glycoprotein B (gB) according to Mitchel *et al.* (1994) were used. The “outer” set was 5'-GTCGACGGTGGAGATACTGCTGAGG-3' and 5'-GAGGACAACGAAATCCTGTTGGGCA-3', which amplifies a 150 bp sequence. The “inner” primer pair was 5'-ACCACCGCACTGAGGAATGT-CAG-3' and 5'-TCAATCATGCGTTTGAGGAGGTA-3', which amplifies a 100 bp sequence.

The material for amplification was 20 ng of DNA isolated from leukocytes. The reaction took place in a thermocycler (Biometra) in buffer containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 1 U of *Taq* polymerase DyNAzyme™ (Finnezymes), 200 μM dNTP and 100 ng of each primer. Samples were amplified for 35 cycles under the following conditions: 94°C for 5 min (first denaturation), 95°C for 2 min, 58°C for external primers and 50°C for internal primers for 2 min, 72°C for 1 min, followed by a final elongation step of 72°C for 7 min. All amplifications were carried out with negative and positive controls. A check for the presence of *Taq* DNA polymerase inhibitors was performed by adding 2 ng of DNA (obtained from a cell culture infected with the AD-169 strain of HCMV) to the negative samples and retesting. The PCR products were visualized by electrophoresis in 2% agarose gel with ethidium bromide.

In situ PCR. *In situ* PCR was done according to the protocol based on Lewis (1996) and Nuovo (1996). The appropriate sample was digested with pepsin (2 mg/ml) for 30 min and afterwards inactivated in 100% ethanol. The reaction was carried out in a thermocycler (Perkin Elmer). A volume of 25 μl of reaction mixture was applied onto the slide. The mixture contained 1 μM primers; 200 μM nucleotides; 20 μM digoxigenin-labeled dUTP (DIG-dUTP, Applied Biosystems); 4.5 or 5 mM MgCl_2 , 0.2 U/ μl polymerase *Taq* IS or Stoffel Fragment and 1 \times buffer (pH 8.3, 100 mM Tris/HCl, 500 mM KCl/10 \times) for IS polymerase or 1 \times buffer (pH 8.3, 100 mM Tris/HCl, 100 mM KCl/10 \times) for Stoffel Fragment. A suitable concentration of magnesium chloride was established, 4.5 mM and 5 mM for IS polymerase and Stoffel Fragment, respectively. In addition to the primers for the gene coding for gB, primers for the gene encoding phosphoprotein pp65 (fluorescently labeled) were also used (Zaia *et al.*, 2001). The “outer” primers were MP1 (5'-CTCG-TAACCACCGAGCGCAAG-3') and AP4 (5'-TCAAC-CTCGGTGCTTTTTGGGCG-3'), the “inner” primers RAP1 (5'-GGATTCCGACAACGAAATCCAC-3') and AP5 (5'-ATACGCTTCCAATTCGGCGAA-3') yielding PCR products of 458 bp in the first run and 171 bp in the second.

For amplification of the target sequences, a modified program of two cycles was used: 94°C for 3 min in the first cycle, and 20 repetitions of 94°C for 45 s and 60°C for 1.5 min in the second cycle (Nuovo, 1996). The hapten, incorporated into the amplified product, was detected using anti-digoxigenin antibodies conjugated to alkaline phosphate. The signal, resulting from the activity of the alkaline phosphate in the presence of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), was obtained according to the manufacturer's instructions (Boehringer Mannheim). The signal resulting from fluorescein was detected under a fluorescent microscope.

In situ RT-PCR. Samples were prepared in the same way as for *in situ* PCR. After pepsin treatment the samples were incubated in DNase solution (1 U/ μl) at 37°C for 12 h. Reaction was carried out in the thermocycler (Perkin Elmer), 25 μl of reaction mixture was applied onto the slide. The mixture contained 1 \times buffer EZ (Applied Biosystems), 4 mM MnCl_2 , 300 μM dNTP, 15 μM DIG-dUTP, 1 μM of each primer specific for gB, 0.2 U/ μl rTth DNA polymerase, which has both polymerase and reverse transcriptase activity (Applied Biosystems), 0.5 U/ μl RNasin (Applied Biosystems). The RT-PCR assay was performed under the following conditions: 65°C for 30 min, preliminary denaturation at 94°C for 3 min, 20 cycles at 60°C for 1.5 min and 94°C for 45 s. Detection was carried out in the same way as for *in situ* PCR.

RESULTS

In 28 samples of peripheral leukocytes, pp65 antigen was present in the cytoplasm (Fig. 1). Among the 12 samples without the pp65 antigen, two were obtained from seronegative recipients without history of HCMV infection. They were used as a negative control. All 40 samples were retested using nPCR, *in situ* PCR and *in situ* RT-PCR. Additionally, we used controls without polymerase or primers, which were helpful in correct interpretation of the results in the "*in situ*" methods. In some cases this evaluation was essential because of the repair ability of DNA polymerase. The results obtained by pp65 antigenemia, nPCR, *in situ* PCR and *in situ* RT-PCR are presented in Table 1. Using nPCR, viral DNA was detected in 27 clinical specimens, seven samples were negative. In the remaining six samples the results were unreadable due to the presence of polymerase inhibitors. The amplification results of these specimens in the presence of 2 ng of DNA positive for HCMV were also negative. In 20 blood samples we detected the viral DNA in the nucleus and/or cytoplasm of investigated cells by *in situ* PCR. In the same samples we found late viral transcripts using *in situ* RT-PCR, which confirmed productive infection. Figures 2 and 3 show examples of the results obtained with *in situ* PCR using different techniques of visualizing positive signal. Figure 2 shows results of *in situ* PCR with primers for the gene coding for the protein gB detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase. Figure 3 shows results of *in situ* PCR with fluorescently labeled primers for the UL83 gene, coding for late pp65 protein. Figure 4 shows result of *in situ* RT-PCR reaction with primers for the gene encoding gB.

In eight pp65-positive samples in which the *in situ* RT-PCR results were negative the presence of CMV antigen in the cytoplasm could probably be due to phagocytosis (the presence of DNA in the cytoplasm was also detected by *in situ* PCR – Fig. 3).

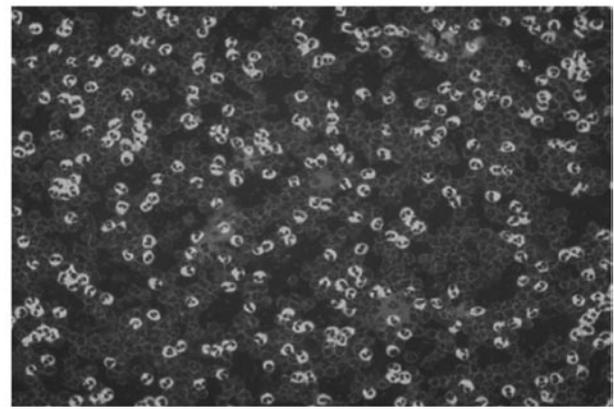


Figure 1. Example of HCMV pp65-antigen presence in the cytoplasm of peripheral blood leukocytes in cytospin preparation-immunofluorescein staining (magnification 250×).

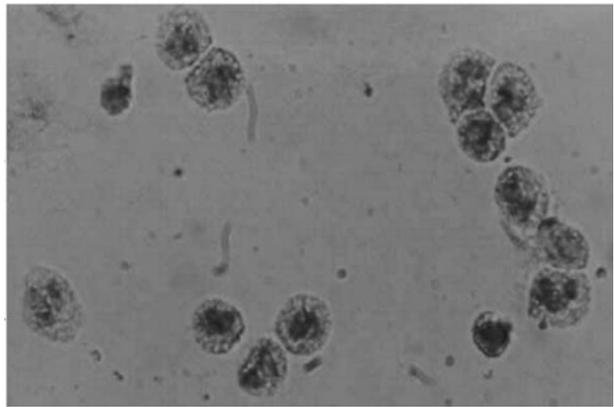


Figure 2. The presence of HCMV DNA in the nucleus of peripheral blood leukocytes obtained by *in situ* PCR with primers specific for gene coding for gB HCMV (magnification 400×).

Among antigenemia-negative specimens, the latent form of HCMV was recognized in five samples on the basis of HCMV DNA presence in the nucleus (Fig. 2) and absence of transcripts for late viral genes (gB) using the *in situ* methods.

Table 1. Results of HCMV DNA or mRNA detection in 40 blood samples obtained by three methods, as related to the presence or absence of pp65 antigen in cytoplasm of leukocytes.

Conclusion of investigation	No. of samples	Ag pp65	nPCR	PCR <i>in situ</i>	RT-PCR <i>in situ</i>
Productive infection	15	+	+	+(N/C*)	+
	5	+	inh**	+(N/C)	+
Phagocytosis	7	+	+	+(C)	-
	1	+	inh	+(C)	-
Latent infection	5	-	+	+(N)	-
Lack of infection	5	-	-	-	-
Negative control	2	-	-	-	-

+, positive result; -, negative result; *signal localization: N, nucleus; C, cytoplasm; **inhibitors of PCR reaction

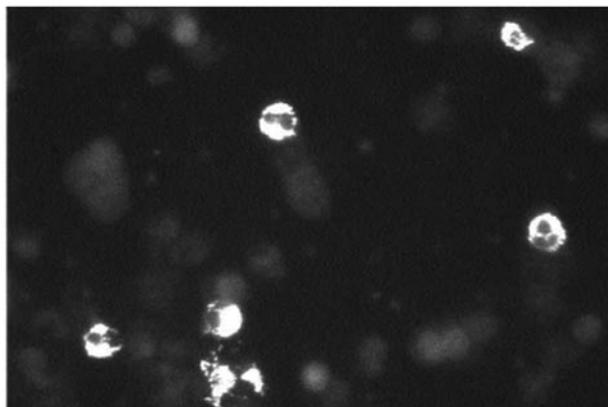


Figure 3. The presence of HCMV DNA in the cytoplasm of peripheral blood leukocytes obtained by *in situ* PCR with fluorescently labeled primers specific for UL83 gene encoding pp65 protein (magnification 400 \times).

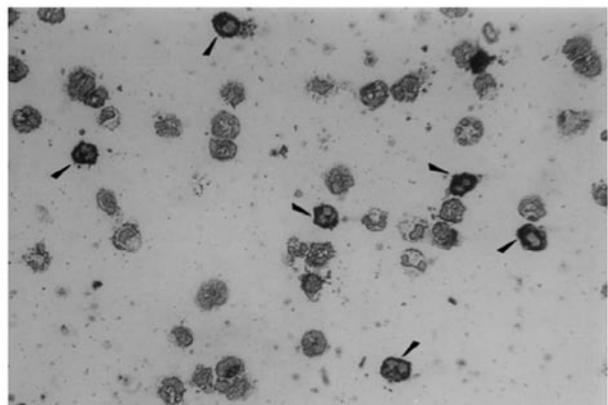


Figure 4. The presence of HCMV transcripts in the cytoplasm of blood leukocytes obtained by *in situ* RT-PCR with primers for gene coding for gB (magnification 400 \times).

DISCUSSION

In immunocompromised patients, especially after organ transplantation, rapid, sensitive and reliable detection of HCMV infection is necessary (Amorim *et al.*, 2001).

Quantitative pp65 – antigenemia assay is regarded as a valuable and sensitive tool for predicting HCMV disease although interpretation of its results is not straightforward. Most instructions recommend recording cells with only nuclear fluorescence. In our prior study such cells were found rarely, mainly in patients with symptoms of HCMV disease, while leukocytes with a positive signal in the cytoplasm occurred quite frequently (Piatkowska-Jakubas *et al.*, 2003). In such cases we used additional tests, *in situ* PCR and *in situ* RT-PCR, which confirmed the results of our antigenemia test and explained the source of the pp65 antigen in the cytoplasm of leukocytes. On the basis of the *in situ* methods, in eight

samples we detected the antigen that could be derived by phagocytosis.

Nested PCR with its high sensitivity is useful as a screening test but this approach cannot discriminate between latent and productive infection. In this situation it should be supplemented with quantitative methods or detection of specific viral transcripts as well as *in situ* methods (Gerna *et al.*, 2000; Kosz-Vnenchak *et al.*, 2001; Yakushiji *et al.*, 2002; Nitsche *et al.*, 2003; Ikewaki *et al.*, 2005).

In situ PCR is the coupling of a PCR assay and nucleic acid hybridization. It allows for detection and visualization of the nucleic acid in question within leukocytes, in the nucleus or cytoplasm (Komminoth & Long, 1995; Kosz-Vnenchak *et al.*, 2001). This is important for confirmation of latent infection, especially when other results are ambiguous. We confirmed five such cases with latent infection, which we used as negative controls. *In situ* PCR makes it possible to achieve high sensitivity, but it may cause non-specific signals as a result of mispriming, DNA repair or diffusion of the product (Komminoth & Long, 1995). Because of this, one should carry out control experiments in order to evaluate the results in a proper way. In using *in situ* methods the main problem was high background.

The presence of viral mRNA is considered the most important indicator of active viral replication (Gozlan *et al.*, 1992; Deiman *et al.*, 2002). In our study, *in situ* RT-PCR detecting late HCMV transcripts, such as mRNA for glycoprotein gB, was used to confirm productive viral infection. In 20 samples we detected active HCMV replication and in 13 specimens phagocytosis or latency was probable. In all these cases *in situ* methods were very helpful. In samples in which it was impossible to carry out nPCR because of the presence of polymerase inhibitors, the *in situ* methods played a crucial role in proper diagnosis.

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