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Minimal Effect of Delayed Sample Processing on Results of Quantitative PCR for Cytomegalovirus DNA in Leukocytes Compared to Results of an Antigenemia Assay

PETER SCHÄFER,[1]* WERNER TENSCHERT,[2] KAI GUTENSOHN,[3] AND RAINER LAUFS[1]

Institut für Medizinische Mikrobiologie und Immunologie,[1] Urologische Klinik,[2] and Abteilung für Transfusionsmedizin,[3] Universitäts-Krankenhaus Eppendorf, 20246 Hamburg, Germany

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Quantitative cytomegalovirus antigenemia and DNAemia were determined in peripheral blood leukocytes of 25 patients stored for up to 72 h at room temperature (RT) and 4°C before processing. Numbers of antigen-positive cells significantly decreased with time. The decline was greater at RT than at 4°C. In contrast, no significant alterations in DNAemia occurred.

The quantitative pp65 antigenemia assay is the method of choice for rapid diagnosis of cytomegalovirus (CMV) infections, prediction of CMV disease, and monitoring of antiviral therapy (1, 8, 9, 17–23). However, it is recommended that specimens be processed within 6 h of collection because delays may diminish pp65-positive cell counts (7, 13, 19), leading to misjudgment of the patients' risk of CMV disease. Recently, the number of CMV DNA copies in peripheral blood leukocytes (PBLs) has also been found to correlate with symptomatic CMV infection and to allow monitoring of antiviral treatment (3, 5, 6, 8, 11, 15, 16, 24, 25). Supposing that CMV DNA in PBLs might be less subject to deterioration than pp65 antigen, the goal of our study was to comparatively evaluate the influence of blood specimen processing delays of up to 3 days on the quantitation of pp65 antigen and CMV DNA.

Twenty-five patients with suspected active CMV infection following renal transplantation were investigated. To exclude factors apart from storage conditions influencing CMV quantitation, only patients without current antiviral therapy were enrolled in the study. Thirty milliliters of blood was freshly collected in EDTA tubes from each participant. Unseparated cells were quantitated with a hematologic counter and adjusted to 10^7 leukocytes per ml with phosphate-buffered saline (PBS), pH 7.4. Adjusted whole-blood samples were dispensed into 50- and 100-μl aliquots and subjected to antigenemia assay and PCR, respectively. Baseline values were determined after immediate processing, and reduction kinetics were established after storage of adjusted whole-blood aliquots at room temperature (RT) and 4°C for 6, 24, 48, and 72 h (Fig. 1) before processing. Despite the small aliquot volumes, which could not be avoided due to the large number of measurements to be made, the same number of cells was analyzed as when cells were obtained from larger volumes. This procedure, therefore, most likely resembled the conditions of delayed blood sample delivery to a laboratory. Moreover, other investigators have reported whole blood to be more stable than initially enriched leukocytes stored in virus transport medium or PBS (2, 12, 14).

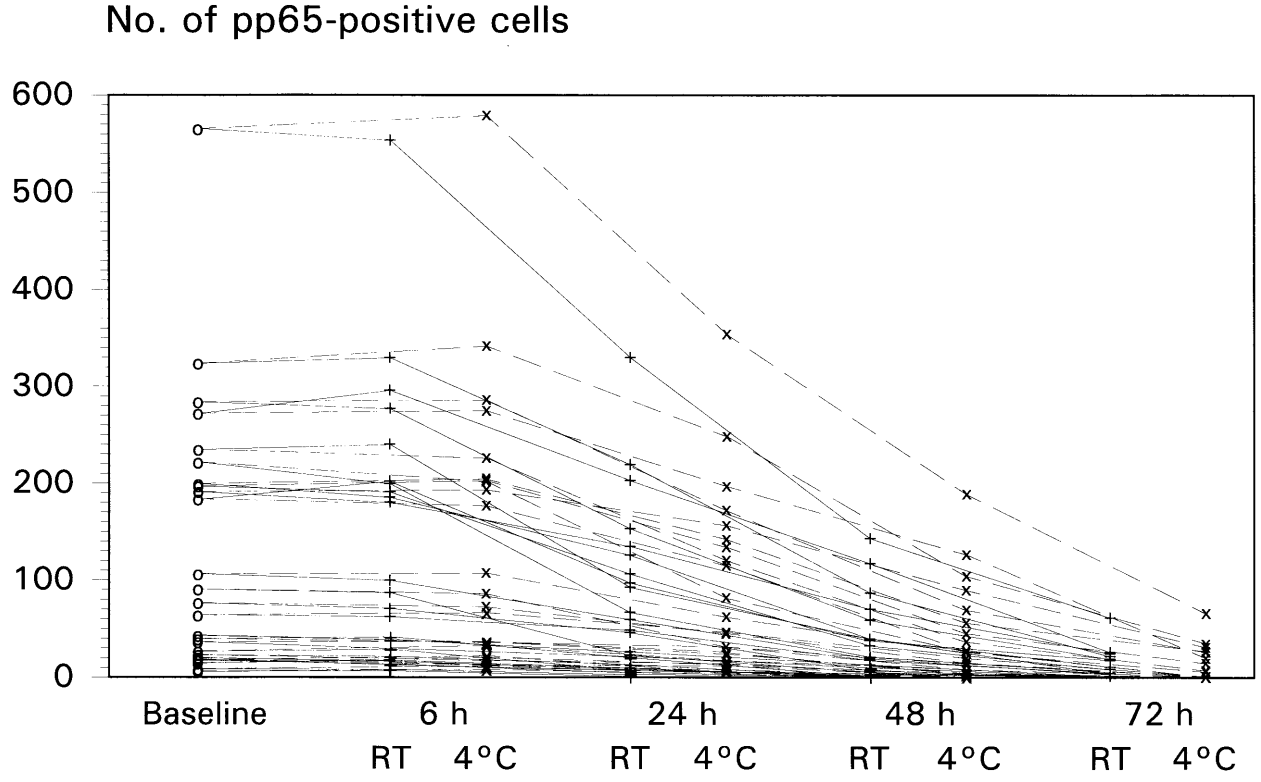
For detection of pp65 antigen, 50-μl aliquots were centrifuged at 700 × *g* for 3 min and erythrocytes were lysed with 0.8% ammonium chloride. Due to the small aliquot volume, initial leukocyte enrichment by dextran sedimentation (7) was omitted. However, this did not influence recovery of leukocytes. PBLs were washed and resuspended in 500 μl of PBS. PBLs (10^5; 100 μl) were centrifuged onto glass slides, fixed, and permeabilized with 5% paraformaldehyde–0.5% Nonidet P-40, and antigen-positive cells were detected by indirect immunofluorescence (7) with Clonab CMV (Biotest, Dreieich, Germany) by following the manufacturer's instructions. Results were expressed as averages of two pp65-positive cell counts from each aliquot with deviations of ≤15% for cell counts of ≥100 and ≤30% for counts of ≤50 (data not shown).

CMV and β-globin target sequences were quantitated by competitive PCR by using cloned standard (st) sequences as previously described (11, 16), with slight modifications. Cells of 100-μl aliquots were centrifuged at 700 × *g* for 3 min and lysed in 0.8% ammonium chloride. Pelleted lysates were washed with PBS, and supernatants were discarded. DNA was extracted from leukocyte nuclei by digestion with 100 μg of proteinase K per ml (10, 16) in a 40-μl reaction volume. After being boiled for 10 min, a 10-μl supernatant sample was added to a PCR mixture (10, 16) to a total volume of 100 μl containing either 5×10^3 copies of the CMV st and 5×10^5 copies of the β-globin st (high st) or 50 copies of the CMV st and 5×10^4 copies of the β-globin st (low st), respectively. CMV target sequences were amplified for 20 cycles with external CMV-specific primers (4). Ten microliters of the external reaction mixture was reamplified in a second round of PCR for 30 cycles with the internal CMV-specific primers and the β-globin-specific primers, respectively. st and wild-type CMV and β-globin PCR amplimers were quantitated by hybridization to a strand-specifically labeled st sequence, separation by temperature gradient gel electrophoresis, and densitometric analysis of autoradiographs (16). The number of CMV copies per 10^6 copies of β-globin was determined in each sample. For CMV DNA copy numbers of $\geq 5 \times 10^2$ or β-globin copy numbers of $\geq 10^5$ in 10 μl, the values from the high-st reaction were used, and for CMV copy numbers of $< 5 \times 10^2$ or β-globin copy numbers of $< 10^5$, those from the low-st reaction were used. Results were expressed as average values of two measurements, which differed by ≤15% (data not shown).

Unlike observations reported by Gerna et al. (7), performance of the antigenemia assay in our study revealed no significant difference between baseline and 6-h counts (Fig. 1A). The reasons for this discrepancy remain unclear, since in our

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Immunologie, Universitäts-Krankenhaus Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. Phone: 0049/40/4717 3157. Fax: 0049/40/4717 4881.

A


No. of pp65-positive cells
600
500
400
300
200
100
0
Baseline
6 h
RT 4°C
24 h
RT 4°C
48 h
RT 4°C
72 h
RT 4°C

B

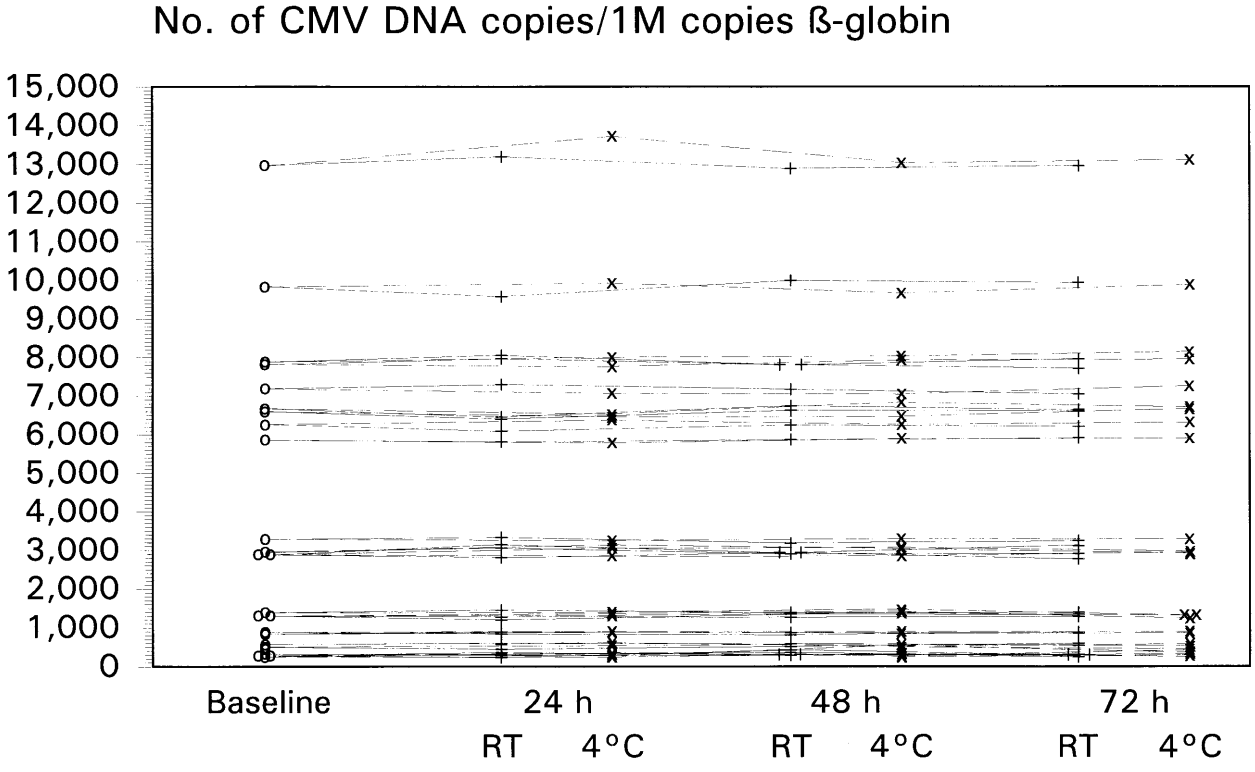

No. of CMV DNA copies/1M copies ß-globin
15,000
14,000
13,000
12,000
11,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
Baseline
24 h
RT 4°C
48 h
RT 4°C
72 h
RT 4°C

FIG. 1. Mean numbers of pp65 antigen-positive cell counts (A) and CMV DNA copies/10^6 β-globin copies (B) determined in duplicate at baseline (○) and after storage at RT (+) and 4°C (×) for 6, 24, 48, and 72 h. (A) Baseline versus 6 h, no statistically significant difference (Wilcoxon's nonparametric test). Differences at 24 h: baseline versus RT, $P < 0.001$; baseline versus 4°C, $P < 0.001$; RT versus 4°C, $P < 0.001$. Differences at 48 h: baseline versus RT, $P < 0.001$; baseline versus 4°C, $P < 0.001$; RT versus 4°C, $P < 0.01$. Differences at 72 h: baseline versus RT, $P < 0.001$; baseline versus 4°C, $P < 0.001$; RT versus 4°C, not significant. (B) Differences between CMV/β-globin ratios were not statistically significant (results at 6 h not shown).

study the same fixation and detection techniques were used. In agreement with other reports (2, 12), we observed significant decreases after storage at 24, 48, and 72 h at both RT and 4°C, with a considerable amount of false-negative test results at ≥48 h. The decline was significantly greater in samples stored at RT than in those stored at 4°C for 24 or 48 h (Fig. 1A), whereas in other investigations (2, 12, 14), differences in positive-cell counts at RT and 4°C after 24 h did not reach statistical significance. However, in these studies results were expressed as percentages of reduction from the baseline value. Statistical analysis based on absolute cell counts (Wilcoxon's nonparametric test) is more meaningful because the absolute number of pp65 antigen-positive cells (ranging from breakpoint values of ≥20 to ≥40/10^5 PBLs) has been shown to correlate with disease activity (1, 6, 8, 9, 17–25). Therefore, the cell declines we observed rendered baseline determination of the antigenemia level necessary. Delayed processing of samples could lead to misinterpretation of the course of infection, since in our setting it was not possible to reliably extrapolate baseline counts of ≤30 positive cells from values obtained on the following days (Fig. 1A).

In contrast, throughout the observation period, CMV/β-globin copy number ratios showed no significant changes (Fig. 1B). Despite the high number of PBLs processed for each PCR in the present study compared with our previous investigations (11, 16), we observed greater fluctuations of absolute CMV DNA measurements than of CMV/β-globin ratios (data not shown). Thus, to rule out errors arising from uneven DNA extraction efficiency, we regard standardization of DNA yield by quantitation of β-globin target sequences to be essential.

In patients with current antiviral therapy, the decline of antigen-positive cells in stored specimens has been reported to be accelerated (12). Such patients were not enrolled in our study because it seemed likely that antiviral substances in stored samples influence quantitation of antigenemia and DNAemia to different extents.

Our findings indicate that no significant decrease in CMV DNA copy numbers in PBLs is measurable by CMV/β-globin PCR for at least 72 h after sample collection, whereas a delay for ≥24 h causes a marked decline in pp65-positive cells. We conclude that quantitative PCR from PBL can be a useful diagnostic tool, especially in outpatient monitoring for CMV relapse, as test validity should be maintained even with specimens shipped by mail. In contrast, samples to be tested by the antigenemia assay should be processed within 6 h postdrawing to ensure valid interpretation of results.

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