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Balance between herpes viruses and immunosuppression after lung transplantation

Verschuuren, Erik Alfons Maria

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Chapter 4

Direct Quantification of Human Cytomegalovirus Immediate-Early and Late mRNA Levels in Blood of Lung Transplant Recipients by Competitive Nucleic Assay Sequence-Based Amplification

Astrid E. Greijer, Erik A. M. Verschuuren, Martin C. Harmsen, Chantal A.J. Dekkers, Henriette M. A. Adriaanse, T. Hauw The and Jaap M. Middeldorp

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Abstract

The dynamics of active human Cytomegalovirus (HCMV) infection was monitored by competitive nucleic acid sequence-based amplification (NASBA) assays for quantification of IE1 (UL123) and pp67 (UL65) mRNA expression levels in the blood of patients after lung transplantation. RNA was isolated from 339 samples of 13 lung transplant recipients and analyzed by the quantitative IE1 and pp67 NASBA in parallel with pp65-antigenemia and serology. Rapid increases in IE1 RNA exceeding 10^4 copies per 100 μ l of blood were associated with active infection, whereas lower levels were suggestive for abortive, subclinical viral activity. Any positive value for pp67 RNA was indicative for active infection, and quantification of pp67 mRNA did not give additional diagnostic information. The onset of IE1-positive NASBA preceded pp67 NASBA and was earlier than the pp65-antigenemia assay, confirming previous studies with qualitative NASBA. Effective antiviral treatment was reflected by a rapid disappearance of pp67 mRNA, whereas IE1 mRNA remained detectable for longer periods. Quantification of IE1 might be relevant to monitor progression of HCMV infection but should be validated in prospective studies.

Introduction

Human Cytomegalovirus (HCMV) is ubiquitous in humans, and primary infection generally occurs without clinical symptoms. However, in AIDS patients, newborns, transplant recipients and other immunocompromised individuals, HCMV can cause severe disease (5). In order to prevent development of HCMV-related disease in these patients, well-timed pre-emptive initiation of antiviral therapy is of importance, guided by an early and accurate diagnosis. Especially in transplant recipients, frequent monitoring for HCMV infection is essential for appropriate patient management, since symptoms of infection and rejection of the transplanted organ may be similar, whereas the therapeutic approaches are opposite (12,23,28). The development of accurate diagnostic approaches has been ongoing for many years, aiming to solve several problems: early detection of aberrant virus activity and discrimination between abortive, subclinical infection and clinically relevant viral activity leading to HCMV disease. Currently, antigenemia monitoring is increasingly used to initiate antiviral therapy (22,25,26) and may be confirmed by shell-vial culture (13), whereas nucleic acid (DNA and RNA) diagnostics are still being validated for their diagnostic relevance in many institutes (3). For the specific detection of HCMV RNA transcripts in patient materials, nucleic acid sequence-based amplification (NASBA) was developed (1). In contrast to HCMV DNA, which may be present in circulating leukocytes as a stable and inert molecule (17), the presence of HCMV specific mRNA directly reflects viral biological activity. Systemic spread of HCMV via productively infected circulating blood leukocytes is a hallmark of disseminating infection, closely linked to development of HCMV disease, and should be limited at an early stage (24). Studies using reverse transcription-PCR for detection of mRNA have indicated that late viral transcripts reflect active HCMV replication in contrast to immediate-early transcripts, which lack specificity for prediction of HCMV disease (11,16,18,20). However, reverse transcription-PCR detecting late mRNA as pp150 (18) and UL18 (14) was only positive in the peak of infection. The low sensitivity may be overcome by using an abundantly expressed mRNA such as pp67 (6).

In recent studies, qualitative NASBA for the detection of late-stage pp67 (UL65) RNA, encoding a structural tegument protein, has proved to be a sensitive and specific assay for monitoring active systemic HCMV infection in solid transplant recipients (1,8). From the study of Blok et al. (1) it was concluded that NASBA for late pp67 mRNA is more sensitive than the antigenemia assay for the detection of HCMV infection in renal allograft recipients. Furthermore, pp67

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NASBA proved useful for monitoring progression of HCMV infection in heart, lung and bone marrow patients and to determine the effect of antiviral therapy with results comparable to those of the antigenemia and DNA-emia assays (8). However, in high-risk bone marrow transplant recipients, the pp67 NASBA showed a mean delay of 2 days before becoming positive in comparison to antigenemia results.

In order to identify active HCMV infection at an earlier stage, an NASBA assay was developed for qualitative detection of mRNA encoded by the immediate early gene UL123 (IE1) (2,9). IE1 NASBA proved to be highly sensitive, detecting the onset of both primary and secondary Cytomegalovirus infection significantly earlier than cell culture, antigenemia, and pp67 NASBA in renal, liver, heart, and lung transplant recipients (2,21). Also in bone marrow transplant patients IE1 NASBA was significantly earlier than pp67 NASBA, pp65-antigenemia and DNA-emia (9). The IE1 NASBA results indicated that IE1 mRNA detection might provide a useful parameter for starting preemptive antiviral treatment in high-risk patients. However, following anti-viral therapy, HCMV IE1 mRNA may still be expressed, since current antiviral drugs selectively inhibit viral DNA replication and thereby late mRNA synthesis, but may leave earlier stages of viral gene expression relatively unaffected. Therefore, the merely qualitative IE1 NASBA may not be ideal for monitoring HCMV activity. The high sensitivity and associated lower specificity for predicting symptomatic HCMV infection of qualitative IE1 mRNA monitoring was further indicated in previous studies using reverse transcription-PCR (16,18,20). This was confirmed by a study of Oldenburg evaluating IE1, β 2.7 (early mRNA) and pp67 gene expression by NASBA in thoracic organ transplant recipients (21). In this study, IE1 mRNA was detected in a significant number of cases with subclinical HCMV infection that did not require antiviral treatment. The early detection of IE1 mRNA in certain samples might be related to HCMV-positive blood transfusion. A quantitative NASBA for measuring IE1 RNA levels could be more informative for accurate monitoring of relevant HCMV activity potentially leading to disease. Currently no data are available on the quantity and kinetics of symptomatic and subclinical HCMV RNA expression in blood during active infection.

To analyze in more detail the in vivo dynamics of mRNA expression in the circulation of HCMV-infected patients and to determine their relevance for predicting the progression of subclinical infection to disease, quantitative versions of the HCMV-specific IE1 and pp67 NASBA assays were developed. Quantification was performed by incorporating a competitively coamplified RNA construct with identical length and sequence as the wild-type (wt) RNA, except for 20 randomized nucleic acids, allowing its specific detection. HCMV RNA

expression levels were determined in weekly samples of unfractionated whole blood in control patients and in lung transplant recipients with primary and secondary HCMV infections by quantitative IE1 and pp67 NASBA and compared to pp65 antigenemia and serology in simultaneously obtained samples.

Materials and Methods

Clinical samples

Heparinized whole-blood samples and serum specimens of patients transplanted between 1997 and 1998 were collected weekly following transplantation during admission and at each patient visit thereafter. One ml blood was mixed with 9 ml of NASBA lysis buffer (5 M guanidine isothiocyanate, 0.1 M Tris [pH 6.4], 20 mM EDTA [pH 8.0], 1.2% [wt/vol] and stored at -80°C. Serum samples were stored at -20°C.

Patients

In the period from 1997 to 1998, 17 patients received a lung transplant, of which 4 patients died within 3 months. Of the 13 lung transplant patients studied, 2 patients were HCMV seronegative and received a seronegative transplant, 3 patients were seronegative and received an organ from a HCMV seropositive donor, and 8 patients were already seropositive, of which six transplant recipients received an HCMV-positive and 2 received a HCMV-negative transplant. The two negative donor/recipient matches did not show any signs or symptoms of HCMV infection. Of the primary infected patients, all three had an active HCMV infection that required antiviral therapy. Of the 8 seropositive patients receiving a positive transplant, 5 developed an active HCMV infection, whereas the two seropositive patients receiving a negative transplant did not have a reactivation of HCMV.

Treatment

All patients received standard immunosuppressive treatment with rabbit anti-thymocyte globulin (3 mg/kg, 2-5 times postoperatively, Thymoglobulin®, Merieux, France), azathioprine (1.5-3 mg/kg/day), cyclosporine A (dose adjusted to whole blood trough levels of 400 µg/l within 3 weeks tapering to levels of 150 µg/l), prednisolone (three times 125 mg the first day, 0.2 mg/kg/day from day 2 to third month and 0.1 mg/kg/day thereafter), pneumocystis carinii prophylaxis with cotrimoxazole (960 mg on alternate days), and acyclovir (200 mg four times a day for 6 months). Acute rejection was treated with pulse therapy of

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methylprednisolone (500-1000 mg intravenously daily for 3 days). Recurrent rejection was treated by replacement of cyclosporine by FK506 (Prograf, Fujisawa, Japan) and subsequently from azathioprine to methylmycophenolate mofetil (cellcept, Roche, Switzerland). Cytomegalovirus-related disease was treated with intravenous ganciclovir (Cymevene, Roche) or Foscarnet (Foscavir) until pp65-antigenemia levels dropped below the limit of detection (25). One patient received hyperimmune globulin, (Megalotect; Biotest, Dreieich, Germany) in addition to ganciclovir and foscarnet.

Antigenemia and serology

HCMV antigenemia was determined as described previously (25,26). In short, peripheral blood leukocytes were isolated by dextran sedimentation and cytocentrifuged onto glass slides. These slides were air dried, fixed with formaldehyde, permeabilized with Nonidet p-40, and stained with a mixture of monoclonal antibodies C10 and C11, directed against the pp65 protein (Biotest) using an indirect immunoperoxidase technique. The total number of HCMV antigen-positive cells was scored and expressed per 50,000 leukocytes analyzed. Immunoglobulin M (IgM) and IgG antibodies against HCMV were determined by a semi-quantitative enzyme-linked immunosorbent assay (ELISA) using alkaline glycine-extracted HCMV antigens obtained from HCMV AD169-infected fetal fibroblasts and in parallel on an extract of mock-infected fibroblasts (27). Serum samples were added in serial twofold dilutions starting with 1:100, and bound HCMV-specific antibody was detected with a peroxidase-labeled sheep antibody against human immunoglobulins. The amount of antibody present in the patient serum was calculated relative to that of a standard serum which was included in each plate. Results were quantitatively expressed as a percentage of the standard sera containing high levels of IgM and IgG antibodies, which were set at 100 U.

Virus and cell culture

Human fetal lung fibroblasts (HLF) were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah). HLF cells were infected with cell-free HCMV virus at 0.01 PFU per cell during 1 hour, and medium with 10% serum was refreshed, followed by incubation for 3 days. Infected cells were washed twice with phosphate-buffered salins (PBS) and harvested by trypsinization, followed by centrifugation at 1,000 x g. The cell pellet was washed with PBS and dissolved in NASBA lysis buffer.

Nucleic acid isolation

Total nucleic acid was isolated from 1 ml whole blood in NASBA lysis buffer using a solid-phase silica extraction technology essentially as described by Boom et al (4). Before isolation, the internal calibrator RNA (Q RNA) was spiked into the sample (10^4 copies of IE1 and 2×10^5 copies of pp67 Q RNA per ml sample). The Q RNA allows accurate and controlled quantification of the amplified target RNA. The Q RNA was identical to the sequence of amplified wt RNA except for the region complementary to the enhanced chemiluminescence (ECL) detection probes (Greijer et al., submitted for publication). The extracted nucleic acids were eluted into 50 μ l of elution buffer and stored at -70°C .

Qt NASBA

The quantitative (Qt) NASBA amplification reaction was performed on 5 μ l of nucleic acid eluate using with two primers, which were designed to amplify a part of the mRNA encoded by UL65 and UL123 exon 4 (1,2). The IE1 and pp67 NASBA was carried out essentially as described by Greijer et al. (submitted). Briefly, wt and Q RNA were coamplified using a T7 promoter containing primer and a reverse primer. The amplification was initiated by an enzyme mix containing avian myeloblastosis virus-reverse transcriptase (Seikagaku, Rockville, Md), RNase H, and T7 RNA polymerase (Pharmacia, Upsala, Sweden) (15). Amplification products were detected by electrochemiluminescence system, using capture probes coupled to magnetic beads and wt- and Q-specific ruthenium-labeled oligonucleotide detection probes by the NASBA QR system (Organon Teknika, Boxtel, The Netherlands). The number of wt RNA molecules per 100 μ l of blood was calculated by a formula based upon a linear regression of the ratio between the wt signal and the calibrator signal (7). For determination of the parameters in the formula, three RNA concentrations of IE1 and pp67 in vitro RNA (10^3 , 10^4 and 10^6 copies per ml blood in lysis buffer) were tested sixfold. From these dose-effect curves, correction factors *a* and *b* were estimated, which were used to calculate wt RNA and Q RNA concentration according to the formula; $\log Wt_{\text{conc}} = 1/a \times (\log WT_{\text{ECL}} - \log Q_{\text{ECL}} + \log Q_{\text{conc}}) - b/a$.

Results

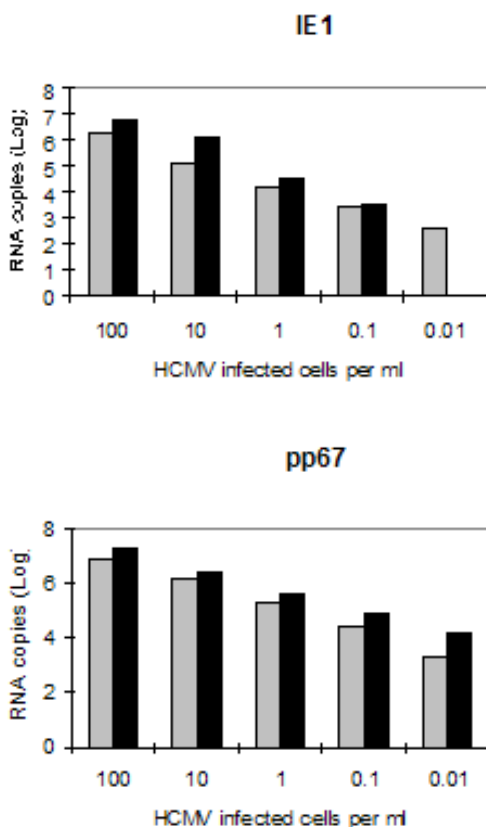
Performance of the IE1 Qt and pp67 Qt NASBA

The analytical performance of the IE1 and pp67 Qt NASBA is described elsewhere (Greijer et al, submitted). The sensitivity of Qt NASBA was similar to

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the qualitative NASBA described earlier, which had a threshold level of 70 copies of IE1 RNA (9) and 100 copies of pp67 RNA. For analyzing the influence of whole blood on the quantification of HCMV RNA, HLF were infected with HCMV at 0.01 PFU per cell for 72 hours cells followed by homogenization with NASBA lysis buffer. The homogenate was diluted to a range of 0.01 to 100 HCMV infected cell equivalents per ml of lysis buffer or spiked at identical concentrations in whole blood from healthy individuals. The sensitivity for pp67 Qt NASBA in both lysis buffer and blood was 0.01 HCMV- infected cell equivalent per ml (Fig. 1). The IE1 NASBA is sensitive to 0.1 cell equivalent per ml due to the 10-fold-lower expression level of IE1 mRNA in infected HLF cells (Greijer et al., submitted), confirming previous findings of Davis et al. (6).

Figure 1: Characterization of IE1 and pp67 Qt NASBA in fibroblasts infected with HCMV at multiplicity of 0.01 in NASBA lysis buffer (shaded bars) compared to cells in a background of 100 μ l of blood from healthy donors in NASBA lysis buffer (solid bars).



The quantification of cells spiked in both lysis buffer and blood was linear over a range of 0.1 to 100 cell equivalents, which is equivalent to a range of 10^3 to 10^6 RNA copies per ml (Greijer et al submitted).

Detection of IE1 and pp67 mRNA in control groups

To determine the clinical relevance of IE1 and pp67 Qt NASBA assays, whole blood samples of healthy individuals were analyzed. The IE1 Qt NASBA did not give any positive results for 144 healthy individuals tested. However, the pp67 Qt NASBA showed two weakly positive results among 144 healthy individuals. Since these results were obtained from seronegative individuals, these low positive values were considered to be due to a contamination. From two HCMV-seronegative transplant recipients receiving an allograft from a seronegative donor, 25 and 28 subsequent samples were analyzed respectively, over a period of 36 and 60 weeks post transplantation. Neither IE1 nor pp67 Qt NASBA showed any positive result.

Monitoring of IE1 and pp67 RNA during HCMV infection

For routine diagnosis of HCMV infection, the pp65-antigenemia assay was performed in a standardized method as described by The (The 98). Results of the pp65-antigenemia were used as an indication to start and terminate antiviral therapy. The IE1 and pp67 Qt NASBA assays were performed retrospectively on samples collected simultaneously with routine pp65-antigenemia testing. Results of pp65-antigenemia, IE1 and pp67 Qt NASBA assays were compared (Table 1). Patients were grouped by primary and secondary infection. Of the 292 blood samples tested, 130 samples (45%) were pp65-antigenemia positive, whereas 188 (65.7%) were positive for IE1 Qt NASBA and 60 (21.0%) were positive for pp67 Qt NASBA.

Most blood samples positive for pp65 antigenemia in patients with a primary or secondary HCMV infection had detectable levels of IE1 RNA (89.2%), whereas pp67 RNA was present less frequently (36.9%), especially in patients with a secondary HCMV infection (21.5%). However, when clinically relevant levels of pp65 antigenemia were detected (>10 pp65-positive cells per 50,000 tested), a higher number of patients with secondary HCMV were also positive for pp67 mRNA (54.5%). Of 80 blood samples with negative results for pp65 antigenemia, detectable levels of IE1 mRNA were found in 41 samples (51.9%) and pp67 mRNA in 7 samples (8.9%). Positive pp67 Qt NASBA results with negative pp65 antigenemia were all located in periods after the first peak of infection, where pp65 antigenemia results fluctuated between low positive and negative. For

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further interpretation, the time to the first positive IE1 and pp67 Qt NASBA results after transplantation was analyzed in more detail.

Table 1: Comparison of IE1 and pp67 NASBA with antigenemia results^a

Infection	Antigenemia Assay	No. of samples	No. (%) of test samples			
			IE1 Qt NASBA		pp67 Qt NASBA	
			Positive	Negative	Positive	Negative
Primary	Positive	51	47 (92.2)	4 (7.80)	31 (60.8)	20 (39.2)
	Negative	26	18 (69.2)	8 (44.4)	6 (23.1)	20 (76.9)
	No result	11	2 (18.2)	9 (81.8)	0 (0)	11 (100)
Secondary	Positive	79	69 (87.3)	10 (12.7)	17 (21.5)	62 (78.5)
	>10 ^b	11	11 (100)	0 (0)	6 (54.5)	5 (45.5)
	Negative	53	23 (43.4)	30 (56.6)	1 (1.89)	52 (65.8)
	No result	66	29 (43.9)	37 (56.1)	5 (7.58)	61 (92.4)

^a Samples with 'no result' for antigenemia are derived from patients who had two successive negative antigenemia results, after which antigenemia was not determined again.

^b Clinically significant threshold values.

Time to detection of HCMV infection

In lung transplant patients with a primary infection, the first repeated pp65 antigenemia positive result was used to initiate antiviral treatment. The timing of the first positive result by the IE1 and pp67 Qt NASBA assays was determined relative to the pp65 antigenemia assay during the early phase of infection. The results are given in Table 2. In patients with a primary HCMV infection, the onset of infection could be detected at the same time (3.4 weeks after transplantation) by pp65 antigenemia and IE1 Qt NASBA. Late pp67 mRNA was detected with a 5-day delay compared to pp65 antigenemia and IE1 Qt NASBA. In patients with a secondary HCMV infection, pp65 antigenemia became positive at a mean 3.6 weeks after transplantation. In two patients, the IE1 mRNA was detected 7 and 10 days earlier than pp65 antigenemia, whereas in the other patients, both assays became positive simultaneously. pp67 RNA was detected with a delay of 1.5 weeks compared to pp65 antigenemia. In this comparison, pp65 antigenemia results of more than one positive cell were used, which is considerably below the clinical level relevant for secondary infection. A patient with a secondary infection received antiviral therapy only where these were more than 10 pp65 positive cells in the antigenemia assay or when clinical symptoms indicated active HCMV infection. Using the latter as criterion for the eight patients with a secondary infection, three patients had a positive result for pp67 mRNA. These patients already had high levels of pp65 antigenemia at the first available sample, and both IE1 and pp67 Qt NASBA were positive at the first tested sample.

Table 2: Time to first HCMV detection in blood of patients with a primary and secondary infection in comparison with the antigenemia assay.

Infection	HCMV assay	No. of patients	Mean time post transplantation (wk) of first HCMV appearance (first result- last result)
Primary	Antigenemia	3	3.4 (1.7-4.4)
	IE1 Qt NASBA	3	3.4 (1.7-4.4)
	pp67 Qt NASBA	3	4.1 (2.7-5.4)
Secondary	Antigenemia	5	3.6 (2.1-6.0)
	IE1 Qt NASBA	7	3.3 (1.6-6.0)
	pp67 Qt NASBA	5	5.8 (3.3-8.0)

Quantification of HCMV IE1 and pp67 mRNA levels in transplant recipients

In order to define the clinical relevance of HCMV RNA quantification, the levels of mRNA encoded by the IE1 and UL65 gene were compared to the level of pp65 antigenemia. For this purpose, pp65 antigenemia results were divided into negative and positive pp65 antigenemia and clinical relevant numbers of pp65-positive cells (Table 3). The mean level of expression of IE1 was log 5.3 RNA copies per 100 μ l of blood. In patients with a primary infection, mean IE1 expression levels rose 19-fold to log 6.6 RNA copies per 100 μ l of blood, paralleled by antigenemia levels exceeding 10 cells per 50,000 polymorphonuclear leukocytes, whereas in patients with a secondary infection IE1 RNA levels did not exceed log 5.8 copies of IE1 RNA per 100 μ l of blood. Quantification of pp67 RNA shows an increase in the number of RNA copies with the number of pp65-positive cells. The overall mean level of pp67 RNA molecules was log 4.5 and the maximum number of RNA copies was log 5.6 per 100 μ l of blood.

The comparison of IE1 and pp67 RNA quantification relative to the number of pp65-positive cells in the pp65 antigenemia assay of the individual blood samples is shown in Fig. 2. The level of IE1 RNA expression correlated with the level of the antigenemia (Fig. 2A), with 0 to 10^3 RNA copies per 100 μ l of blood being associated with negative antigenemia results. The level of pp67 RNA also showed correlation with increasing antigenemia levels, although much less stringent (Fig. 2B).

In the current routine diagnostic approach, the course of HCMV infection in lung transplant recipients is reflected by the number of pp65-positive cells in the antigenemia assay. In order to acquire additional information on the progression of HCMV infection, the kinetics of IE1 and pp67 mRNA expression in blood were analyzed. The antigenemia, IE1 and pp67 mRNA levels of four representative

patients are presented in Fig. 3. For evaluation of the immune response, the levels of HCMV-specific IgM and IgG antibodies are presented as well. Fig. 3A and B represent two lung transplant recipients with a primary infection. In Fig. 3C and D represent two patients with a secondary HCMV infection.

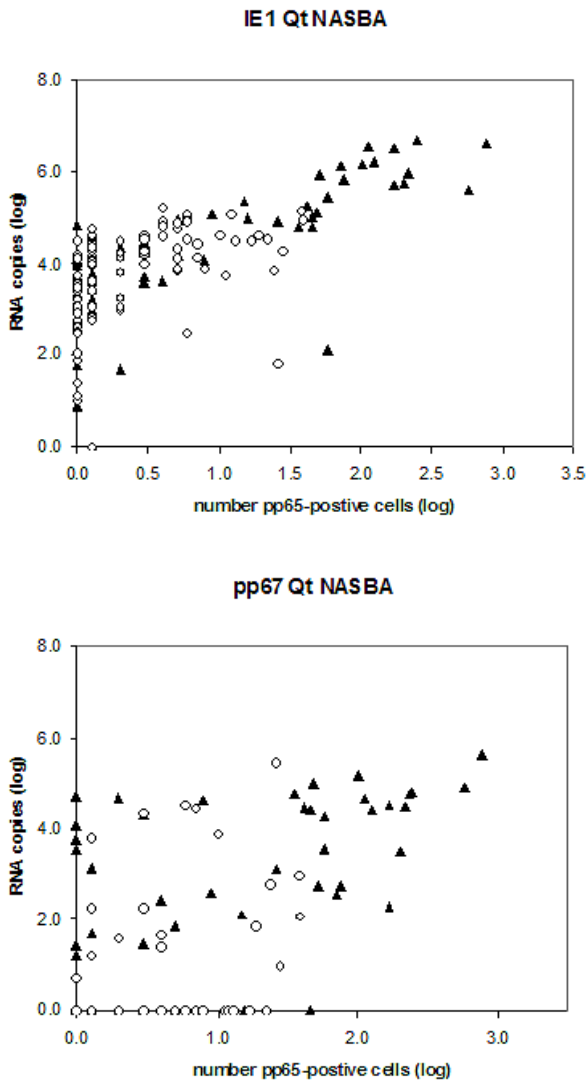
Table 3: Comparison of RNA levels with antigenemia results in 11 transplant recipients (153 SAMPLES) with a primary and secondary HCMV infection

Infection	Antigenemia (No. of positive cells)	IE1 Qt NASBA		pp67 Qt NASBA	
		No. Positives	RNA copies (log)	No. positive	RNA copies (log)
Primary	0	18	3.89	6	4.12
	1	9	3.97	2	2.85
	2-10	13	4.77	7	4.20
	>10	23	6.04	22	4.71
Secondary	0	24	3.55	1	0.71
	1	20	4.21	3	3.32
	2-10	35	4.58	8	4.04
	>10	11	4.66	6	4.62

Kinetics of IE1 and pp67 mRNA levels in blood of patients with HCMV infection

Patient 1 was seronegative and received a positive lung transplant. In order to prevent acute rejection, the patient was treated four times with methylprednisolone. The onset of HCMV infection was detected by antigenemia at 12 days after transplantation. At the same moment, IE1 Qt NASBA became positive, whereas the pp67 Qt NASBA became positive at 19 days. Three periods of active infection were seen. During the second and third peak of infection, the number of positive cells in the antigenemia assay increased considerably, to a maximum of 640 positive cells. Therapy with ganciclovir was successful at first but proved ineffective in the second period, and was therefore replaced by foscarnet. Sequencing of the viral DNA isolated from samples at week 24 and 25 revealed a C→T (Ala→Val) mutation at codon 594 of the UL94 gene, known to cause ganciclovir resistance.

Figure 2: Levels of pp65-positive cells in the antigenemia assay compared with levels of IE1 and pp67 RNA levels determined by Qt NASBA in lung transplant patients with primary (\blacktriangle) and secondary (\circ) HCMV infection.



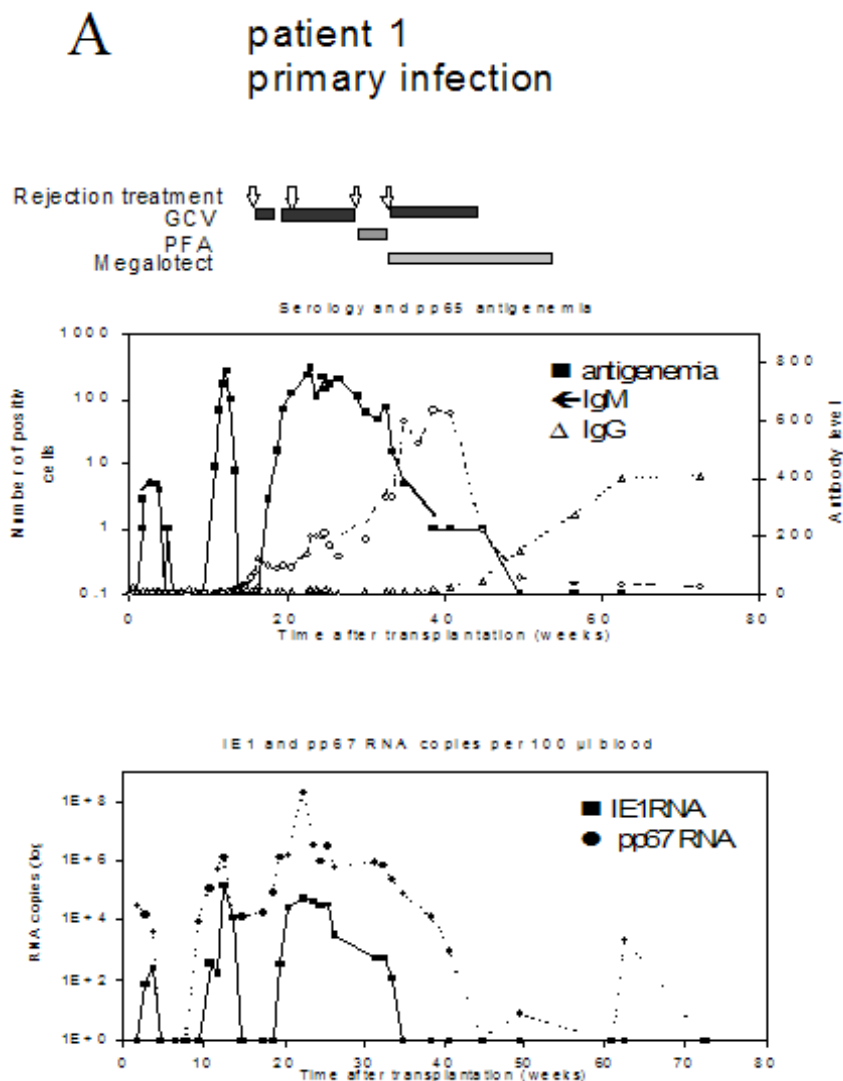
Due to adverse side effects, foscarnet treatment was ended 15 weeks after transplantation, and ganciclovir in combination with Megalotect was given until the antigenemia levels had decreased significantly. Fig. 3A clearly shows that at the initial phases of viral activity, the dynamics of IE1 and pp67 RNA expression were similar to the antigenemia. However, IE1 RNA levels remained positive

Figure 3: Course of HCMV infection in four representative patients by serology, pp65-antigenemia, and IE1 and pp67 Qt NASBA assays.

(A and B) Patients with primary HCMV infection.

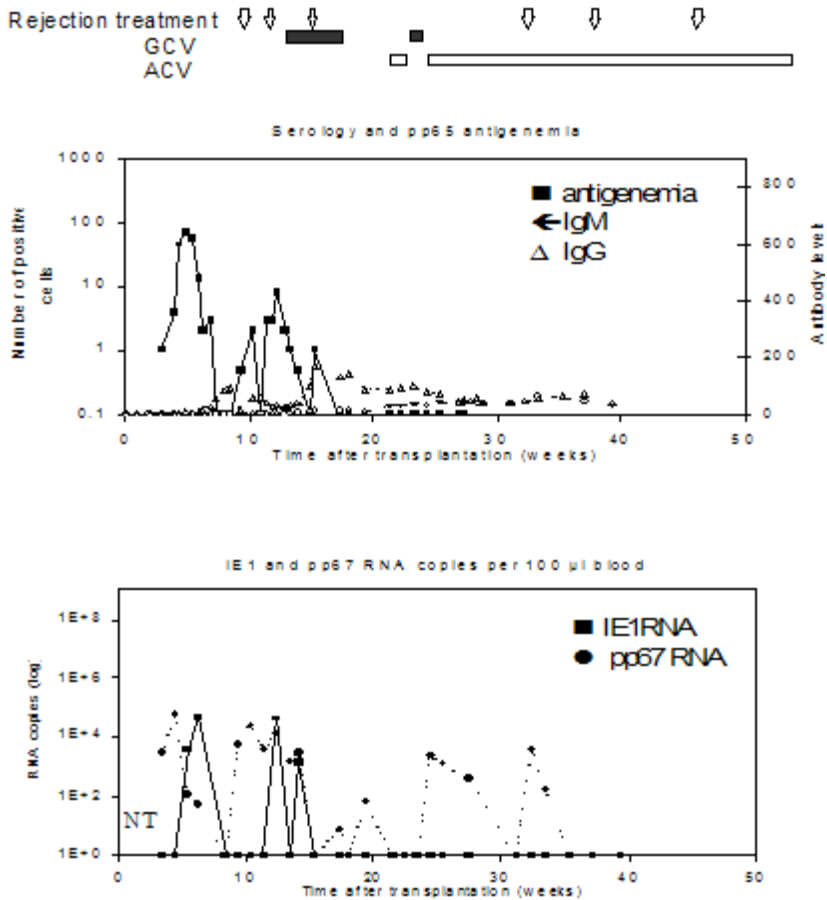
(C and D) patients with secondary HCMV infection.

GCV, ganciclovir; PFA, foscarnet; ACV, acyclovir. Arrows, rejection treatment



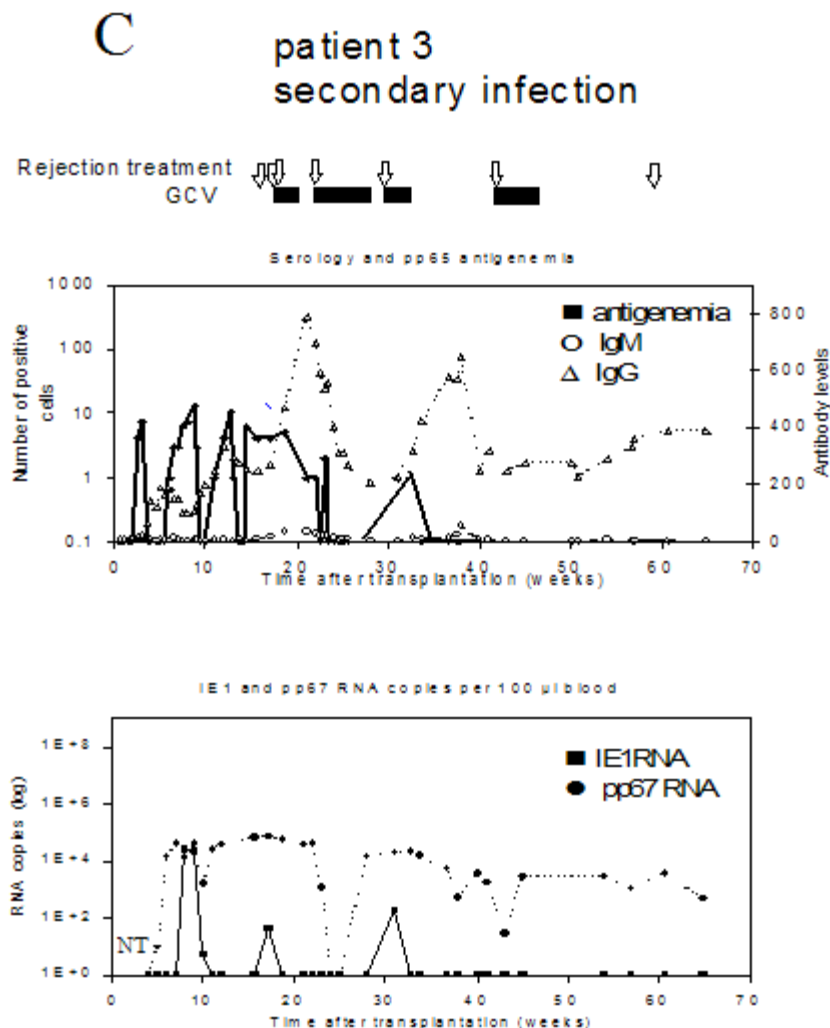
during the period of foscarnet therapy, while antigenemia and pp67 Qt NASBA were negative. The pp67 Qt NASBA detected HCMV infection in all three periods of active infection 1 week later than IE1 NASBA. During therapy with foscarnet, pp67 RNA and antigenemia were negative. Significantly decreased pp67 RNA

B patient 2 primary infection



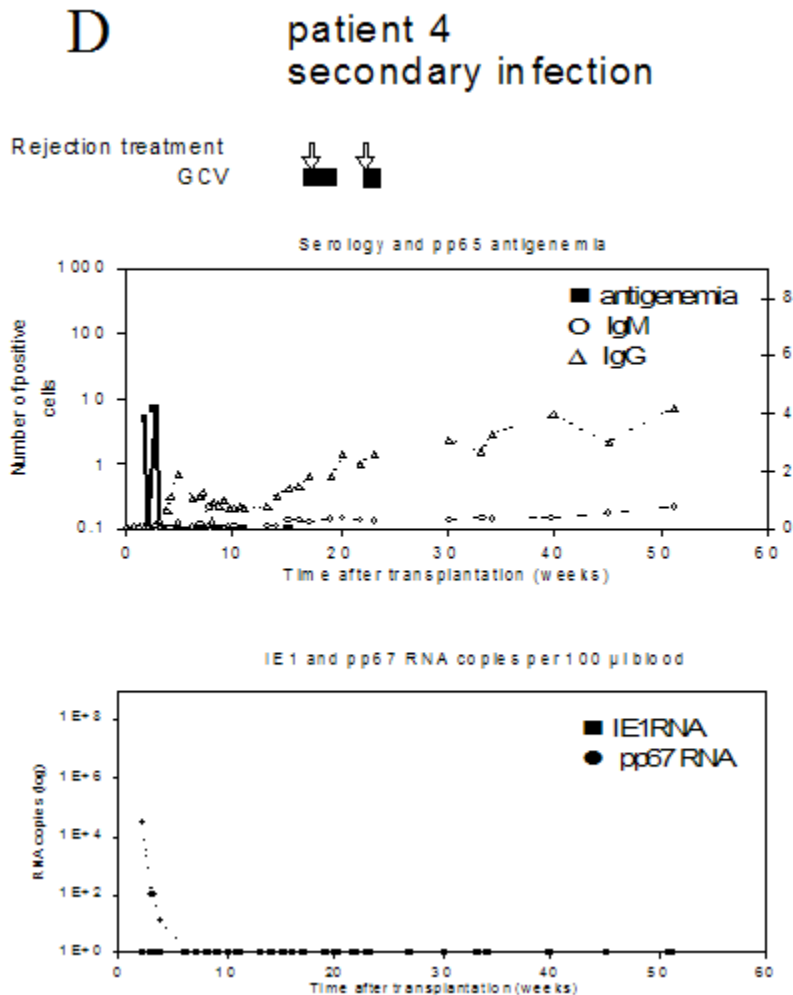
levels indicated the final success of therapy at 14 weeks, which was before antigenemia and IE1 RNA.

Patient 2 had a primary HCMV infection and subsequently developed a primary Epstein-Barr virus (EBV) infection. Antirejection therapy was given six times. The acyclovir treatment for EBV is also shown in figure 3B. After 4.4 weeks, increasing antigenemia showed the first signs of HCMV infection. Simultaneously, IE1 Qt NASBA became positive, and IE1 RNA levels corresponded with the antigenemia levels. pp67 RNA was detected with a delay of 7 days and weeks later in the second peak of infection. In contrast to patient 1, the levels of



was 3 IE1 RNA expression were substantially lower. Furthermore, IE1 RNA reappeared each time a rejection treatment was given, while the antigenemia and the pp67 Qt NASBA assay remained negative. The sole presence of IE1 RNA indicated restricted activity of the HCMV infection, since clinical symptoms of HCMV infection were absent (subclinical infection).

An example of the kinetics of IE1 and pp67 RNA expression in a HCMV-seropositive transplant patient is shown in figure 3C. Patient 3 had seven rejection treatments and four periods of HCMV activity, with short antiviral treatments with ganciclovir. Although antigenemia did not exceed 20 pp65-positive cells, ganciclovir was given in order to prevent progression of the



infection during antirejection therapies. The onset of HCMV infection was defined at 18 days after transplantation. Prior to this period, samples were not available for analysis of IE1 and pp67 mRNA. In the second peak of infection, antigenemia and IE1 Qt NASBA were positive simultaneously at 6 weeks, whereas pp67 Qt NASBA was positive with a 2-week delay. Levels of IE1 RNA remained elevated during periods of positive antigenemia, and when antigenemia and pp67 became negative, IE1 RNA was still detectable, although at lower levels. The pp67 Qt NASBA showed positive results only in the highest peaks of infection.

Patient 4 was seropositive and received an HCMV positive organ. A secondary infection was diagnosed at 12 days after transplantation, and

antigenemia was detectable for 1 week (Fig. 3D). The first positive result by antigenemia could not be tested by NASBA, but the second sample was positive for both antigenemia and IE1 Qt NASBA. The pp67 Qt NASBA did not show any positive results in the period of elevated IE1 RNA level. The low positive antigenemia did not necessitate antiviral therapy, but in combination with rejection therapy, ganciclovir was given prophylactically.

Discussion

Active HCMV infection remains a significant problem in the transplantation population. Control of HCMV activity in solid organ transplant recipients is of importance for preventing HCMV disease and improving overall clinical performance. The main implication of our study is that quantification of pp67 RNA does not provide additional information compared to the previously described qualitative pp67 RNA detection, whereas quantification of IE1 RNA more directly reflects the dynamics of HCMV infection. Furthermore, the rapid increases to high levels of IE1 RNA are associated with progression to disease. Previous studies have shown that qualitative monitoring for the presence of HCMV RNA by either reverse transcription-PCR or NASBA could provide a useful diagnostic approach directly reflecting active viral gene expression in circulating HCMV-infected cells (11,16,18,20). In recent years, monitoring for the presence of pp67 and IE1 mRNA by NASBA was introduced to provide a new approach for early detection of systemic active infection. The pp67 NASBA has a relatively limited sensitivity, since it detects only the late productive stage of infection. However, pp67 mRNA monitoring may provide timely diagnosis for initiation of preemptive therapy (1,8,21). IE1 NASBA, although providing very early positive results, may lack specificity for predicting disease (2,9,21).

In order to monitor patients more closely and analyze the kinetics of expression of IE1 and pp67 in more detail, quantitative competitive pp67 and IE1 Qt NASBA assays were developed. In healthy control blood donors and HCMV-seronegative patients receiving an HCMV-seronegative organ, IE1 and pp67 mRNA could not be detected by the Qt NASBA assays at any time during follow-up. Of the lung transplant recipients tested, three primary infected patients had active, symptomatic HCMV infection, whereas of the eight seropositive transplant receptions, five had an active and partly subclinical infection. The two seropositive patients receiving a negative organ did not develop HCMV infection, suggesting that the infections in the former may be derived from the donor organ and therefore were defined as secondary infection. Local cytokine responses related to early rejection episodes might be responsible for triggering HCMV

reactivation in the transplanted organ (19). The time of the first detection of IE1 and pp67 RNA by Qt NASBA in the lung transplant recipients is comparable to the qualitative findings by Blok et al. (2) and Gerna et al (9), as expected, since both qualitative and quantitative NASBA assays showed, comparable levels of sensitivity for in vitro RNA (9;Greijer et al, submitted).

The mean time to detect a positive result for IE1 Qt NASBA in lung transplant recipients was 3.4 weeks, compared to 3.7 weeks in kidney transplants (2) and 5.4 weeks in bone marrow transplant recipients (9). The mean time of the first detection of pp67 RNA was at 4.1 weeks post-transplantation in primary infected patients and 5.8 weeks in transplant recipients with secondary infection, compared to 5.1 weeks in kidney transplant recipients and 6.2 weeks in bone marrow patients. The primary infections were detected at an earlier stage compared to patients with a secondary infection. The peak levels of RNA in 100 μ l of blood of lung transplant recipients were log 6.6 molecules of IE1 RNA and log 5.6 molecules of pp67 RNA.

In the blood of patients with an active HCMV infection, pp67 RNA levels are lower than IE1 RNA levels, which contrasts to the observation in infected HLF cells in vitro (log 4.7 copies of IE1 RNA and log 5.3 copies of pp67 RNA per infected cell) (Greijer et al., submitted). However, in vivo the HCMV RNA expression levels in circulating cells may be different from cultured cells and may differ for different cell types as well. Therefore it is important to note that levels in whole blood represent an averaged value of HCMV RNA expression.

In addition, the higher levels of IE1 RNA may be explained by high numbers of cells with a restricted or abortive infection, expressing only immediate-early RNA without pp67 RNA expression, or by a difference in the mRNA expression ratios in blood leukocytes compared to HLF cells (10).

When monitoring patients with antigenemia and IE1 and pp67 Qt NASBA, pp67 RNA was detected only in the highest peak of infection, during the period in which the antigenemia assay shows medium and high levels of positive cells.

Immediate-early RNA was often present at levels up to 10^4 copies before positive antigenemia results are seen. After treatment with ganciclovir or foscarnet, pp67 levels decreased rapidly, whereas IE1 RNA remained detectable for longer periods. The prolonged detection of IE1 RNA reflects the limitation of current antiviral therapy, which interferes only with the late phase of the viral replication cycle, leaving the expression of IE1 unaffected. The final disappearance of IE1 RNA may indicate full immunological recovery and may suggest long-term control over HCMV infection. This, however, remains to be determined in subsequent studies.

Our results suggest that the dynamics of the IE1 RNA level are useful for monitoring disease progression. IE1 RNA levels up to 10^4 were detected in patients with subclinical HCMV infection, and rapidly increasing levels equal to or above 10^4 were related to disease development. pp67 RNA is detectable at a productive infection, though not detectable in the early phases of viral replication, when clinical symptoms are still absent. The number of pp67 RNA molecules did not relate to the number of pp65-positive cells in the antigenemia assay. This perception indicates that the presence and not the level of pp67 RNA is of prognostic value for antiviral drug management. However, it should be realized that antiviral treatment guided by early pp65 antigenemia may have influenced the pp67 mRNA levels detected in this study. The simultaneously pp67 and IE1 mRNA levels by Qt NASBA could provide additional clinically relevant information on the biological activity and progression of HCMV infection in vivo. Especially in primary infected transplant recipients, initiation of antiviral therapy at the first activation of the virus would improve the outcome of HCMV infection, since the host's immune system might not be able to establish a rapid and appropriate response to HCMV. However, the early initiation of antiviral therapy may prevent appropriate antigen stimulation of the immune system, allowing relapse of HCMV replication upon cessation of treatment. On the other hand, the increase and decrease of IE1 RNA could give an indication of the extent to which the HCMV infection is controlled by the host's immune system. Therefore, the pp67 NASBA, indicating active replicating HCMV, in combination with a quantitative IE1 mRNA result might be preferred for optimal patient management.

The results of this study indicate that combined testing for quantitative IE1 RNA and qualitative pp67 RNA can recognize ongoing subclinical HCMV infection, allowing fine-tuning of the period of antiviral therapy to prevent progression to clinical significant HCMV infection. It is now possible to study the effect of treatment intended to prevent all HCMV activity, which may improve long-term allograft outcome. Since antigenemia results were used for guiding antiviral therapy, the clinical relevance of patient monitoring by IE1 Qt NASBA and qualitative pp67 needs to be determined by prognostic studies.

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