Quantification of Cytomegalovirus DNA in BAL Fluid*
A Longitudinal Study in Lung Transplant Recipients

Gerdt C. Riise, MD, PhD; Rune Andersson, MD, PhD; Tomas Bergström, MD, PhD; Agnete Lundmark, BSc; Folke N. Nilsson, MD, PhD; and Sigvard Olofsson, PhD

Study objectives: Cytomegalovirus (CMV) infection is common in patients receiving solid organ transplants, and it is associated with increased morbidity as well as risk for development of chronic rejection. A rapid and sensitive diagnostic method would improve the therapeutic management of CMV infection, including the monitoring of treatment effects. We investigated whether longitudinal determinations of CMV DNA quantities in BAL fluid could be useful for this purpose.

Design: CMV DNA levels in 340 BAL samples from 35 consecutive lung transplant recipients were studied during a median of 18 months. Seventeen (49%) of the patients developed CMV disease with pneumonitis. Twenty-seven CMV disease episodes were diagnosed.

Results: Patients with CMV disease had a significantly higher mean level of CMV copies per milliliter BAL fluid (1,120 ± 4,379) compared with those without (180 ± 1,177, p < 0.01). Viral load as well as acute rejection requiring treatment (≥ A2) were independent risk factors associated with CMV disease. Differences between the groups concerning HLA-DR matching, basic immunosuppressive therapy, and CMV serologic status D/R –/+ vs D/R +/+ were not significant. A diagnostic definition of normality based on the mean level of all episodes without CMV disease +2 SD would discriminate only 9 of the 27 CMV episodes.

Conclusions: Although the viral load is increased during episodes of clinical CMV disease in lung transplant recipients, the quantitative PCR assessment of CMV DNA in BAL fluid is not discriminative enough to be useful as a diagnostic tool for CMV disease.

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Key words: BAL; cytomegalovirus; lung transplantation; quantitative polymerase chain reaction

Abbreviations: CMV = cytomegalovirus, D = donor; PCR = polymerase chain reaction; R = recipient; TBB = transbronchial biopsy

Infection with cytomegalovirus (CMV) is common in patients receiving solid organ transplants, and it is associated with increased morbidity and mortality in this patient group. It is a clinical problem particularly in lung allograft recipients, in whom CMV disease has been associated with the development of bronchiolitis obliterans syndrome and chronic rejection.1

Rapid diagnosis of CMV infection is of great clinical importance, and today direct antigen detection and molecular techniques have largely replaced the slower traditional culture-based techniques. So far, the most promising method has proven to be the polymerase chain reaction (PCR).2–4 A positive CMV PCR response in transbronchial lung biopsy specimens (TBB) has been shown to precede the morphologically manifest CMV disease by 2 weeks in lung transplant recipients.5 Quantitative PCR assessments of CMV DNA load both in urine and blood samples from kidney transplant recipients have showed promising results in diagnosing patients at risk of infection.6,7 However, a problem with the quantitative PCR method has not been a too low sensitivity, but rather how to determine the clinically relevant level of the CMV DNA content. In a recent study, quantitative PCR analysis used in lung transplant recipients accurately measured the amount of CMV load in both tissue and BAL specimens, but...
also showed a large intersubject and intrasubject variability.\(^8\) It is therefore likely that longitudinal studies should be the method of choice to address the question of whether viral load can be used to predict development of CMV disease.

Our intention was to evaluate in a longitudinal analysis whether CMV DNA quantities in BAL fluid were associated with clinically manifest CMV disease and whether this analysis could be used to predict CMV disease.

**Materials and Methods**

**Subjects**

Thirty-five consecutive patients undergoing single lung (\(n = 17\)), bilateral lung (\(n = 11\)), or heart-lung (\(n = 7\)) transplantation from September 1994 to November 1996 were studied longitudinally.

In 30 cases, donors (D) and recipients (R) were matched for CMV serologic status (4 D/R –/– and 26 D/R +/+). In five cases, CMV– organs were given to CMV+ recipients (5 D/R –/–). All CMV serologically positive recipients received prophylaxis consisting of IV ganciclovir 5 mg/kg body weight twice daily for 2 weeks, followed by 5 mg/kg body weight once daily for another 2 weeks. CMV+ recipients received prophylaxis against herpes simplex virus with peroral acyclovir 200 mg four times daily for 4 weeks.

All organs were harvested in a similar fashion. Surgical procedures, immunosuppression therapy, and treatment of acute rejections were performed as described earlier.\(^9\) For detailed patient characterization, see Table 1.

The study design had been approved by the ethical committee at the University of Göteborg, and all subjects gave their consent after both written and oral information.

**Postoperative Follow-up**

Surveillance bronchoscopy with TBB and BAL was performed according to protocol at 2, 4, 8, and 12 weeks, and at 6, 9, 12, 18, and 24 months after transplantation. Additional TBB and BAL were performed approximately 4 weeks after augmented immunosuppressive treatment of rejection episodes, or whenever clinical variables indicated a deterioration in patient status.

Fiberoptic bronchoscopy was done via the oral route after oropharyngeal lidocaine anesthesia and IV propofol sedation. Supplemental 100% oxygen was delivered nasally at a rate of 4 to 5 L/min with blood oxygen saturation continually monitored with a pulse oximeter (Ohmeda; Louisville, KY).

A histopathologic diagnosis of rejection was based on assessment of TBB and BAL samples. The evaluation of acute rejections followed the recommendations of the Lung Rejection Study Group of the International Society of Heart Lung Transplant,\(^11,12\) and chronic rejection with obliterator bronchiolitis according to the grading system established by Cooper et al.\(^3\)

BAL analysis included direct microscopy for CMV inclusion bodies; *Pneumocystis carinii*, fungi, and mycobacteria. Immunochemical techniques for *P carinii*, CMV, and *Legionella pneumophila* in BAL or TBB were applied routinely. Cultures for bacteria, including Legionella, and mycobacteria, fungi, and virus

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<th>Table 1—Clinical Characteristics and CMV Serologic Status of Lung Transplant Recipients With and Without CMV Pneumonitis*</th>
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<tr>
<td><strong>With CMV Disease</strong></td>
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<tr>
<td>Preoperative Diagnosis</td>
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<td></td>
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<tr>
<td>Alpha-1 AT</td>
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<td>Alpha-1 AT</td>
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<td>Alpha-1 AT</td>
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<tr>
<td>Alpha-1 AT</td>
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<tr>
<td>Emphysema</td>
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<td>Eisenmenger syndrome</td>
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<td>PPH</td>
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<td>PPH</td>
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<tr>
<td>PPH</td>
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<tr>
<td>Cystic fibrosis</td>
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*\(n = 17\) Median 47 13F/4M 3 months \(n = 18\) Median 44 10F/8M

\(\text{Tx} = \text{transplantation; } \text{Alpha-1 AT} = \text{antitrypsin deficiency; } \text{PPH} = \text{primary pulmonary hypertension; } \text{IPF} = \text{idioopathic pulmonary fibrosis; } \text{SL} = \text{single lung; } \text{BL} = \text{both lungs; } \text{HL} = \text{heart-lung.}\)
were performed, and presence of CMV and respiratory syncytial virus genome was investigated by PCR amplification. CMV disease was defined as clinical symptoms of pneumonia together with presence of CMV inclusion bodies in TBB or BAL samples after the criteria for CMV disease of the Fourth International Cytomegalovirus Workshop. Diagnosis of bacterial infection was based on the presence of significant bacterial growth in BAL sample (> 10⁷ cfu/mL). Diagnosis of P carinii pneumonia was based on demonstration of the organism by immunostaining or silver staining of TBB and BAL samples.

Collection of Samples

All bronchoscopies were performed between 8:30 and 11:30 AM. BAL was performed by infusion of 6 × 20 mL of warmed sterile pyrogen-free phosphate-buffered saline solution into a segmental middle lobe or lingula bronchus with the bronchoscope in a wedged position. The fluid was aspirated after each 60-mL infusion, pooled in a sterile siliconized container, and immediately transported on ice to the laboratory. Cellular components were sedimented by centrifugation at 4°C, 500×g for 10 min.

Cytocentrifuge slides (Shandon Southern Products Ltd; Runcorn, UK) were made from 100-µL aliquots of the resuspended cell pellet. Slides were immediately fixed in 96% alcohol and stained with May-Grünwald and Giemsa for later identification of cell types on a morphologic basis. Percentages of polymorphonuclear granulocytes, eosinophil granulocytes, lymphocytes, and macrophages were calculated by counting 200 cells using a standard light microscope.

TBB specimens were routinely taken after BAL. At least five macroscopically adequate biopsy specimens were sampled under fluoroscopic guidance from different sites within one lung using alligator forceps, immediately placed in 10% buffered formalin, and sent for histopathologic analysis.

Virus Isolation

Volumes of 100 µL each of BAL specimens were inoculated into two cultures of human diploid fibroblasts (cell culture tubes 13 × 120 mm; 2 mL Eagle’s minimal essential medium without additives other than penicillin and streptomycin) and incubated at 37°C under gentle rotation. The cultures were observed for 4 weeks; cultures exhibiting suspected CMV cytopathic effects (plaques of enlarged cells) were confirmed by subjecting 50 µL of the supernatant to CMV PCR as previously described.

CMV PCR Analysis

Quantitative CMV PCR was analyzed by the Viral Quantitative CMV Detection Kit (BioSource Europe, S.A.; Fleurus, Belgium) according to the manufacturer’s instructions for quantitation of CMV DNA in BAL specimens. Briefly, BAL CMV DNA and a plasmid-based internal calibration standard (500 copies/reaction mixture) were amplified, using primers specific for a 254-bp amplicon in a conserved region of the CMV gB gene, in a thermal cycler (denaturation at 94°C, annealing 60°C, extension 72°C; 40 cycles). The amplified DNA was denatured and subsequently captured by probes (one specific for the internal calibration standard amplicon and the other one specific for the authentic CMV amplicon) coated onto the cavities of microtiter plates. After 2 h, specific absorbance values corresponding to bound internal calibration standard and CMV amplicon were recorded, and the number of CMV copies in the specimen was calculated. Specimens giving rise to absorbance values outside the limits recommended by the manufacturer were reprocessed according to the instructions.

Table 2—Serologic CMV Matching, Degree of HLA-DR Mismatching, Number of Diagnostic Procedures, and Diagnostic Outcome in the Two Groups With and Without CMV Disease*

<table>
<thead>
<tr>
<th>Variable</th>
<th>With CMV Disease (n = 17)</th>
<th>Without CMV Disease (n = 18)</th>
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<tbody>
<tr>
<td>CMV match</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D/R −/−</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>D/R −/+</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>D/R +/+</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>HLA A + B mismatch (median)</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>HLA DR mismatch (median)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number of TBB + BAL</td>
<td>157</td>
<td>183</td>
</tr>
<tr>
<td>CMV diagnosis</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Acute rejection at least A2</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td>BOS</td>
<td>3</td>
<td>2</td>
</tr>
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*Statistical comparisons by Mann-Whitney U test. BOS = bronchiolitis obliterans syndrome.

Statistical Analysis

Clinical characteristics are expressed as medians and range. Numerical data are stated as the arithmetic mean ± SD. Results are expressed as the arithmetic mean ± SEM in the longitudinal graphs. Analysis of variance with Fisher’s protected least significant difference post hoc correction was used to analyze for possible differences in CMV DNA levels over time. Cross-sectional analysis of differences between the patient groups was performed with the Mann-Whitney U test. Covariations between BAL cells and number of CMV copies were analyzed with the Spearman rank correlation test.

To adjust for repeated measures over time, logit models for longitudinal data were used, with the intercept regarded as random and the regression coefficients as fixed. Multivariate logistic regression analysis was performed using the glmfit macro provided by the SAS Institute Inc. The dependent variable was CMV disease, and the independent variables were CMV viral load, acute rejection, and levels of immunosuppressive treatment, both cyclosporine and steroid dosage; p values < 0.05 were considered to be statistically significant.

RESULTS

Of the 35 patients in the study, 16 were studied for a period of 2 years, 7 for 18 months, and 10 patients for 12 months after surgery (median, 18 months). Two patients died within 7 months after surgery; one of multiorgan failure and the other of malignant lymphoma. A total of 340 protocol and diagnostic bronchoscopies were performed, with an average of eight episodes per patient within the first postoperative year.

Altogether 27 episodes of CMV disease were diagnosed, 26 as CMV pneumonitis and one as GI CMV. Seventeen of the 35 patients (49%) developed CMV pneumonitis, the majority within 3 months after undergoing transplantation (range, 2 to 24 months; Table 1). Three of the five patients with
CMV D/R −/+ developed CMV disease compared with 14 of 26 patients with D/R +/+ (Table 2). The four patients with D/R −/− did not develop CMV disease (Table 2). Five patients developed bronchiolitis obliterans syndrome, with three belonging to the group with diagnosed CMV pneumonitis.

No statistically significant differences in percentages of BAL neutrophils, eosinophils, lymphocytes, or macrophages were found during episodes with CMV pneumonitis compared with no CMV (Table 3). Percentages of BAL lymphocytes correlated weakly with BAL levels of CMV copies (p < 0.01, r = 0.30).

All patients with CMV disease received ganciclovir 5 mg/kg body weight twice daily for 2 weeks as initial therapy. In spite of antiviral therapy, seven patients suffered a relapse in CMV disease (six with pneumonitis and one in the GI tract). Three of the patients had CMV pneumonitis diagnosed on three different occasions (Table 1). Adjunctive therapy with high-dose IV immunoglobulin (0.5 g/kg body weight on five occasions) was given if CMV disease reappeared, and in one case, foscarnet therapy was instituted. All patients were eventually successfully treated for their CMV disease.

**CMV Disease With Relation to Rejection and Immunosuppression**

Assessment of concurrent acute rejection when CMV pneumonitis was present in TBB specimens was not attempted in the study. The number of acute rejections at least the International Society for Heart and Lung Transplant grade A2 in the group with

<table>
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<tr>
<th>CMV Status</th>
<th>Neutrophils, %</th>
<th>Eosinophils, %</th>
<th>Lymphocytes, %</th>
<th>Macrophages, %</th>
</tr>
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<tbody>
<tr>
<td>CMV (n = 26)</td>
<td>10.7 ± 19.0</td>
<td>0.5 ± 0.2</td>
<td>10.9 ± 9.4</td>
<td>77.8 ± 18.1</td>
</tr>
<tr>
<td>No CMV (n = 238)</td>
<td>11.3 ± 16.4</td>
<td>0.1 ± 0.5</td>
<td>10.5 ± 10.5</td>
<td>78.0 ± 18.2</td>
</tr>
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*Episodes with concomitant acute rejection, bronchiolitis obliterans syndrome, or other infections are excluded. Results are given as mean ± SD.

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**Table 3—BAL Differential Cell Counts During Episodes With and Without CMV Pneumonitis**

**Figure 1.** Longitudinal presentation of mean BAL fluid levels of CMV copies (log scale) during the first postoperative year (mean ± SEM). Black circles denote patients with diagnosed CMV disease (n = 17). Open circles denote patients without CMV disease (n = 18). TX = transplantation.
CMV disease (n = 19) was not statistically different from those of the group without CMV (n = 29, Table 2). The number of treatments with high-dose steroids for postoperative reperfusion injury, alveolitis, or acute rejection did not differ between the groups (33 vs 35 occasions). The degree of HLA-DR mismatching between the groups was similar (Table 2). The basic immunosuppressive levels of cyclosporine and steroid dosage were comparable between the groups.

**CMV Disease With Relation to Quantitative CMV PCR in BAL Fluid**

Overall, the group that developed CMV disease had a significantly higher mean level of CMV copies/mL BAL fluid (1,120 ± 4,379) compared with those without (180 ± 1,177; p < 0.01 by analysis of variance; Fig. 1). This was true also if the four D/R –/– patients in the CMV– group were excluded from the analysis (216 ± 1,289; p = 0.016).

A subanalysis at 2 months after transplantation showed the same pattern, with a mean level of 1,034 ± 2,554 CMV copies/mL BAL in the group with CMV disease vs 118 ± 470 in the group without. The levels increased at 3 months, with the CMV group having a mean of 5,096 ± 9,351 CMV copies/mL BAL vs the group with no diagnosis of CMV 1,098 ± 3,937 CMV copies/mL BAL. At 4 months, when the majority of the CMV infections had been treated, the levels were down to 112 ± 227 CMV copies/mL BAL in the CMV group vs 12 ± 25 CMV copies/mL BAL in the group without CMV. The negative value at 8 months belonged to a single patient who was CMV D/R –/– and never developed a CMV DNA titer over time (Fig 1).

The 27 episodes with diagnosed CMV disease had a mean level of 5774 ± 9310 CMV copies/mL BAL fluid, whereas the 313 episodes without CMV disease had a mean level of 169 ± 953 CMV copies/mL BAL fluid (p < 0.001; Fig 2). This comparison remained significant if the D/R –/– patients were excluded (187 ± 1,003 CMV copies/mL BAL fluid; p < 0.001). If, for diagnostic purposes, one would define a range of normality based on the mean level of all the episodes without CMV disease + 2 SD, it would discriminate 9 of the 27 episodes with CMV, giving a sensitivity of only 33%.

Multivariate logistic regression analysis revealed that independent risk factors for CMV disease were viral load (p < 0.002; odds ratio, 1.3) and acute rejection at least A2 (p < 0.003; odds ratio, 4.0) in the same sampling episode. High-dose steroid treatment, which was given both on occasions of reperfusion injury and alveolitis as well as for acute rejection treatment, was not (odds ratio, 0.2). The probability for CMV disease at a given level of CMV

**Figure 2. CMV copies (log. scale) in BAL fluid from episodes with diagnosed CMV disease (n = 27) and from episodes without CMV disease (n = 313). Data are presented as box plots displaying the median value (50th percentile) and the corresponding 10th, 25th, 75th, and 90th percentiles on either side of the median, as well as the outlying values of the analyzed variables. For BAL episodes without CMV, the lower 50th percentile bar indicates that > 50% of the BAL samples were PCR negative.**

CMV copies per mL BAL (log. scale)

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\[ \text{CMV copies per mL BAL (log. scale)} \]

\[ \text{p < 0.001} \]

CMV disease

No CMV disease
DNA in BAL fluid is illustrated in Figure 3, which shows that a 50% probability of disease is reached at 17,900 CMV copies/mL BAL, and a 90% probability is reached at 24,900 CMV copies/mL BAL. The influence of acute rejection with at least A2 shifts the curve to the left, and a 50% probability of disease is reached at 13,500 CMV copies/mL BAL, and a 90% probability at 20,500 CMV copies/mL BAL (Fig 3).

Virus Isolation

Six samples were excluded because of bacterial superinfection. CMV was isolated in 11 BAL fluid samples, of which 6 coincided with clinical diagnosis of CMV pneumonitis. In four cases, the CMV virus isolation had no temporal connection with CMV disease, and in one case, the positive isolation preceded the CMV pneumonitis by 4 weeks. In altogether 20 episodes of clinical diagnosis of CMV pneumonitis, no CMV was isolated from the BAL fluid. In five samples, herpes simplex virus was isolated, and in three, respiratory syncytial virus.

DISCUSSION

This longitudinal study of 35 lung transplant recipients shows that although the viral load is increased during episodes of clinical CMV disease, the method of quantitative PCR assessment of CMV DNA in BAL fluid is not discriminative enough to clearly separate patients with and without CMV disease.

A total of 49% of our patients developed CMV disease, and in patients receiving solid organ transplants, a range from 60 to 100% has been reported to acquire CMV infection in the posttransplantation period. A major factor contributing to these high numbers is the unavoidable immunosuppressive regimen, which lowers host T-cell immune defenses long-term. We found that viral load and acute rejection of at least A2 were independent risk factors associated with CMV disease, whereas differences between the groups concerning HLA-DR matching and basic immunosuppressive therapy were not associated with an increased risk. We also did not see any difference between CMV D/R +/+ and CMV D/R –/+ concerning the risk for developing CMV disease or suffering relapse after the initial treatment. The diagnostic utility of a positive BAL viral culture was poor, which is in accordance with other authors.3,18,19 No specific pattern in BAL cell differential counts was associated with CMV infection, but BAL percentage of lymphocytes correlated weakly with levels of CMV DNA, a finding of uncertain clinical relevance. However, we did not assess the amount of CMV in the donor organ at the time of transplantation, which has been implicated as a possible predictor of CMV disease.8

The four patients who were D/R –/– did not develop CMV disease, nor did they display detectable BAL quantities of CMV DNA at any time after transplantation. The group who developed CMV disease had a significantly elevated number of CMV copies per milliliter of BAL fluid, but the longitudi-
nal pattern showed that the overlap in number of CMV copies between the two groups at any single time makes the power of the analysis to predict CMV disease very low (Fig 1). This is consistent with earlier findings in lung transplant recipients, whereby a considerable variability in BAL CMV DNA quantities has been found.\(^8\) It is possible that variability in BAL cell return could contribute to some of this. Assessment of the systemic CMV load in recipients of solid organ transplants with CMV disease has shown that they consistently have higher antigenemic burden than asymptomatic patients. It was also evident that patients with heart and liver allografts have higher systemic CMV antigenemia levels than kidney transplant recipients.\(^4\) It seems likely that a direct determination of the viral load in the target organ would be the most sensitive indicator of CMV disease, but an earlier study in lung transplant recipients could not find that a positive BAL culture correlated with diagnosis of CMV pneumonitis.\(^20\) A more recent study evaluating the DNA content in unseparated leukocytes both in BAL and blood found that the positive predictive value for CMV disease was only 50% in blood and 67% in BAL in lung transplant recipients.\(^18\) The authors therefore could not recommend that antiviral treatment should be based only on positive PCR results in BAL, even if a positive result could be an indication of closer patient surveillance. Also, quantitative assessment of CMV in different BAL cell types could be of interest from a theoretical point of view.

Interestingly, we observed that patients who did not develop CMV disease nevertheless could have a seemingly spontaneous occurrence of high amounts of CMV DNA in BAL that then became negative after some months of observation. This finding may indicate that CMV, at least in some patients, may replicate in the lung tissue and achieve surprisingly high titers without causing any clinical symptoms of ongoing CMV disease. For these patients, our data suggest that during a period often exceeding several months, even massive virus replication in the lung tissue is not a decisive pathogenic factor per se. This possible compartmentalization of the infection might be related to a restriction of the viral dissemination by a functioning immune response in spite of immunosuppressive therapy. The kinetics of viral DNA quantities in BAL samples as shown in Figure 1 suggest a cyclic course of the infection both in recipients who develop CMV disease and those who remain healthy. The periodicity of the cycle seems to be 2 to 3 months and may reflect a continuously shifting balance between viral replication and immune responses, and possibly antiviral treatment, in the patients with CMV disease.

In earlier studies on kidney transplant recipients, we have shown that viremia detected in the form of CMV DNA present in serum samples correlated well with clinical disease.\(^21\) Recently, by the use of quantitative PCR, a significant correlation between maximum viral load in blood and CMV disease in this patient category and in liver transplant recipients was reported.\(^22,23\) A consequence of these works and the present study is that quantitative CMV detection in other types of specimens such as serum or plasma or WBCs may be more instrumental for decisions regarding initiation of antiviral treatment as well as for evaluation of treatment effects. Furthermore, in the future search for factors driving the pathogenesis of CMV disease in lung transplant recipients, assessments of the innate and CMV-specific immune responses in BAL\(^24\) and blood might be required to be analyzed in conjunction with quantification of the viral load.

**References**