CMV infection of the renal allograft is much more common than the pathology indicates: a retrospective analysis of qualitative and quantitative buffy coat CMV-PCR, renal biopsy pathology and tissue CMV-PCR

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Abstract

Background. Quantitative blood polymerase chain reaction (PCR) for cytomegalovirus (CMV) is used to direct therapy in kidney transplant patients, but cytomegalic inclusions are rarely found in allograft renal biopsies even with an elevated serum creatinine and apparent CMV disease. The relationship between quantitative blood CMV and renal allograft pathology is unknown.

Methods. Thirteen biopsy samples were available for analysis from patients suspected of CMV disease, who had a buffy coat CMV-PCR drawn within 2–5 days of a renal allograft biopsy for an elevated creatinine. All were evaluated for CMV pathologically, by light microscopy, immunohistochemistry, in situ hybridization and tissue PCR.

Results. Qualitative and quantitative buffy coat CMV-PCR were positive in 10/13 (77%) patients. Tissue CMV-PCR was positive in five (50%) biopsies, including two with CMV inclusions and three with no inclusions. Quantitative buffy coat CMV-PCR levels did not correlate with detection of CMV inclusions in renal tissue. Paradoxically, quantitative buffy coat CMV-PCR was low (239 and 538 copies/µg of DNA) when CMV inclusions were detected. All five biopsies with acute rejection were associated with CMV viraemia and two of the five with allograft CMV inclusions. A quantitative buffy coat CMV-PCR of <100 copies/µg of DNA ruled out disease with CMV inclusions.

Conclusions. CMV nephropathy is much more common than previously reported when sensitive techniques are used for detection in tissue. Acute rejection and CMV viraemia occur commonly together in patients at risk for CMV. Quantitative buffy coat CMV-PCR does not correlate with the presence of CMV inclusions. These findings have implications for management of patients who have elevated serum creatinine and are at risk for CMV disease.

Keywords: biopsy; blood; CMV-PCR; histopathology; kidney; transplant

Introduction

The great majority of renal transplant recipients have latent cytomegalovirus (CMV) infection [1], which in 20–60% of patients causes CMV disease with clinical signs and symptoms of fever, leucopenia and organ involvement [2]. The main reservoir of CMV is white blood cells, but the virus has been detected in most tissues in the body where it remains latent. CMV replication produces immediate early and late antigens directing viral DNA replication and structural nucleocapsid proteins, respectively [3]. Rising anti-CMV IgG titres elicited by these antigens have been considered adequate for diagnosis of CMV infection, but transplant recipients may fail to produce such antibodies. Modern methods targeting DNA replication, such as shell vial techniques and polymerase chain reaction (PCR) are considered preferable and more sensitive to serology [4]. However, these tests are not always conclusive for allograft kidney involvement or management of CMV infection. For example, the sensitivity of CMV-PCR is very high, raising the possibility that a positive test may not signal active disease. Quantitative buffy coat PCR has, in part, improved
the predictive value of this method [5], as we and others have found that CMV-DNA levels exceeding 500 copies/μg of total DNA from peripheral blood leucocytes correlate with symptomatic disease in most cases [3,5]. Some of our patients, however, with lower viral burdens were also symptomatic, while others with high levels of viral DNA were not, exemplifying the controversy as to which is the optimal test of CMV infection [3].

CMV inclusions on renal biopsy are unquestionably direct evidence of tissue invasiveness or CMV nephropathy. These inclusions, however, are detected in < 1% of transplant biopsies of patients with CMV disease [5]. Although it is intuitive that the greater the quantitative load of blood CMV-PCR, the greater the likelihood of CMV inclusions in renal tissue of patients with CMV disease and nephropathy, the relationship between quantitative blood CMV and CMV renal allograft pathology has not been systematically investigated. Few studies thus far have used tissue PCR to detect CMV in renal allograft biopsies. No studies have correlated quantitative buffy coat (blood) PCR with tissue, histopathology, and tissue PCR.

Gnann et al. [6] using primers for the late antigen region detected amplified CMV products by Southern blotting in a small number of biopsies and nephrectomies. All tissue PCR-positive patients had CMV infection proven by viral culture and seroconversion. In that study, patients who were CMV seropositive pre-transplant, but did not have evidence of active infection (viraemia, antigenaemia or DNAemia) after transplantation, were kidney tissue PCR negative, suggesting that tissue CMV-PCR may have immediate clinical application. Others have confirmed that in clinically symptomatic patients, CMV DNA sequences are often present in human allograft biopsies which are negative for CMV in renal tissue by in situ hybridization (ISH) [7]. Thus, the purpose of this study was to correlate qualitative and quantitative buffy coat CMV-PCR with renal allograft tissue histopathology and renal allograft tissue CMV-PCR.

Methods

For this retrospective study, 13 biopsy samples were available for tissue CMV-PCR from patients suspected to have CMV disease. All patients had a buffy coat CMV-PCR drawn within 2–5 days of the renal allograft biopsy obtained for evaluation of an increased serum creatinine. All were evaluated for CMV pathologically, by light microscopy, immunohistochemistry (IH) and ISH. All patients were CMV seropositive pre-transplantation. The CMV serostatus of the donor and recipient are shown in Table 1. No recipient had received ganciclovir or other anti-CMV medications for prophylaxis or treatment prior to the biopsy and CMV analyses.

Haematoxylin and eosin (H&E), periodic acid Schiff-stained slides and immunoperoxidase stains using antibody to CMV immediate-early antigen (Dako, Carpinteria, CA) were retrospectively assessed. ISH was performed in samples from the paraffin block biopsies. Transplant lung tissue with abundant CMV inclusions was used as a positive control. CMV DNA probe (Kreatech, Netherlands) sequences coding for immediate-early antigens were applied and a modified protocol as we have described previously [8]. Briefly, deparaffinized slides, rehydrated through graded alcohols and washed in PBS were treated with Proteinase K (2.5 μg/ml; Sigma, St Louis, MO) in pre-warmed PBS for 30 min. Slides were then acetylated, washed in 2× standard saline citrate (SSC), dried at 55°C for 1 h and hybridized with digoxigenin-labeled probe overnight. The next day the slides were soaked in 5× SSC with 50% formamide at 50°C, followed by RNase (Sigma)

**Table 1. CMV status, symptoms, signs, tissue, blood quantitative CMV-PCR and allograft pathology**

<table>
<thead>
<tr>
<th>Patient</th>
<th>CMV D/R</th>
<th>Symptoms/signs</th>
<th>Qualitative blood PCR</th>
<th>Quantitative blood PCR (copiesDNA)</th>
<th>Qualitative tissue PCR</th>
<th>Pathology</th>
<th>IH</th>
<th>ISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pos/pos</td>
<td>Fever</td>
<td>Pos</td>
<td>239</td>
<td>Pos</td>
<td>CMV inclusions/acute rejection Banff IIA</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>2</td>
<td>Pos/pos</td>
<td>Leucopenia</td>
<td>Pos</td>
<td>598</td>
<td>Pos</td>
<td>CMV inclusions/acute rejection Banff IIA</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>3</td>
<td>Pos/pos</td>
<td>None</td>
<td>Pos</td>
<td>1976</td>
<td>Pos</td>
<td>Interstitial inflammation</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>4</td>
<td>Neg/pos</td>
<td>Fever</td>
<td>Pos</td>
<td>6010</td>
<td>Pos</td>
<td>Interstitial inflammation</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>5</td>
<td>Pos/pos</td>
<td>Fever</td>
<td>Neg</td>
<td>0</td>
<td>Neg</td>
<td>Interstitial inflammation</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>6</td>
<td>Pos/pos</td>
<td>Fever</td>
<td>Pos</td>
<td>345</td>
<td>Pos</td>
<td>Glomerular thrombosis</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>7</td>
<td>Neg/pos</td>
<td>Chronic fatigue</td>
<td>Pos</td>
<td>10</td>
<td>Neg</td>
<td>Glomerular thrombosis</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>8</td>
<td>Pos/pos</td>
<td>Fatigue</td>
<td>Pos</td>
<td>1032</td>
<td>Neg</td>
<td>Acute rejection Banff I B</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>9</td>
<td>Pos/pos</td>
<td>Fever, leucopenia</td>
<td>Pos</td>
<td>6630</td>
<td>Neg</td>
<td>Acute rejection Banff IIA</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>10</td>
<td>Neg/pos</td>
<td>None</td>
<td>Pos</td>
<td>34</td>
<td>Neg</td>
<td>Acute rejection Banff I B</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>11</td>
<td>Pos/pos</td>
<td>None</td>
<td>Pos</td>
<td>276</td>
<td>Neg</td>
<td>Glomerular leucocytes</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>12</td>
<td>Pos/pos</td>
<td>Leucopenia</td>
<td>Neg</td>
<td>0</td>
<td>Neg</td>
<td>Minimal pathologic findings</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>13</td>
<td>Pos/pos</td>
<td>Fever</td>
<td>Neg</td>
<td>0</td>
<td>Neg</td>
<td>CMV inclusions</td>
<td>Pos</td>
<td>Pos</td>
</tr>
</tbody>
</table>

Controls

- TMD Neg No pathologic abnormality
- Lung Pos CMV inclusions

Pos, positive; Neg, negative.
treatment and detection with anti-digoxigenin (DIG; Boehringer Mannheim, Indianapolis, IN) antibody linked to alkaline phosphatase. The reaction was stopped with Tris–EDTA buffer, slides were rinsed and cover-slipped over aqueous mounting medium.

Buffy coat and tissue (extracted as described below) CMV-PCR were performed on DNA extracted from 100 000 blood leucocytes (1 μg of DNA) in all patients and paraffin blocks, respectively. Similar to the ISH, the positive control was obtained from transplant lung tissue with numerous CMV inclusions. The negative control was obtained from four medical renal biopsies with the diagnosis of thin basement-membrane disease. DNA extraction was performed as follows. Between six and eight, 8–10 μm thick sections were cut from each block. One millilitre of xylene was added to each sample twice for 5 min, followed by 100% alcohol dehydration twice for 5 min. The samples were vortexed and centrifuged for 5 min at 12 000 g. The precipitate was lyophilized for 15 min then, 180 μl of DNA buffer and 20 μl of Proteinase K were added overnight at 55°C. The following morning, samples were boiled for 5 min and centrifuged for 5 min at 12 000 g. Five microlitres of DNA was transferred to a clean Eppendorf tube containing 50 μl of distilled H2O. DNA was measured spectrophotometrically (at 260/280 A) and stored at 20°C. The CMV-PCR was performed as we have previously described [3]. Briefly, the assay for detection of CMV was constructed to amplify a 139 bp segment of the CMV late gene product pp65 [9]. The PCR reaction mix consisted of 10 mm Tris pH 8.0, 10 mM MgCl2, 50 mM NaCl, 400 mM of nucleotide triphosphate, 0.2 mg/ml bovine serum albumin, 1 pmol of each primer, and 1 μl of Taq polymerase. The reaction was performed in a Perkin-Elmer 9600 therma cycler, and consisted of 95°C for 2 min, followed by 15 cycles of 95°C for 39 s and 65°C for 60 s. After 15 cycles, the reaction was supplemented with 20 pmol of each primer and 1 μl of Taq polymerase and heated to 95°C for 2 min followed by 32 cycles as described above (booster PCR). The reaction products were analysed in a 3% agarose gel and visualized by ethidium bromide staining and ultraviolet illumination. A band of ~140 bp was visualized with control DNA. Appropriate precautions to avoid contamination were followed. Reactions were considered valid only if positive and negative controls gave the expected results. CMV levels were measured by quantitative-competitive PCR as previously described [3]. Briefly, the quantitative competitive PCR reaction employed an external and an internal standard. The external standard consisted of a cloned 139 bp amplicon from the CMV late gene encoding pp65 [9]. The internal standard was also cloned and consisted of the same amplicon with an internal deletion that preserved the primer binding sites but allowed the resulting PCR amplicon to be differentiated on the basis of size from the amplicon resulting from amplification of the external standard. A standard curve was generated by amplifying 50, 100, 500, 1000, 5000, 10 000 and 50 000 copies of the external standard along with 750 copies of the internal standard. The resulting products were analysed by gel electrophoresis and the amplicon bands quantified using a digital imaging system (IS-1000; Alpha Innotech, San Leandro, CA). Samples were analysed by spiking 750 copies of the internal standard into the PCR mix, and comparing the imaged PCR results with the standard curve. The limit of detection was approximately 50 copies of CMV DNA. The coefficient of variation was 33% at a copy number of 10 000 and 68% at a copy number of 100.

Results

The comparison of renal biopsy pathology with symptoms, signs, qualitative and quantitative blood CMV-PCR is presented in Table 1. The DNA blots are shown in Figure 1.

All patients were CMV-seropositive before transplantation, and 10/13 (77%) received a transplant from a CMV-seropositive donor. All patients had an elevated serum creatinine at the time of biopsy and this was the indication for biopsy. Fever and leucopenia
were the most common symptoms and signs of CMV disease. Overall 10/13 (77%) of those suspected of CMV disease had a positive buffy coat CMV-PCR. The quantitative buffy coat CMV-PCR level in two recipients (patients 7 and 10) with positive qualitative buffy coat CMV-PCR was very low, 10 and 34 copies μg of DNA, respectively.

Histopathologic abnormalities included CMV inclusions, thrombotic microangiopathy, interstitial inflammation, acute rejection, and minimal pathologic changes. CMV inclusions were present in 2/10 (20%) biopsies of patients with CMV viraemia (Figure 2A).

Both cases with CMV inclusions had positive buffy coat CMV-PCR. Viral DNA copies were 239 copies μg of DNA and 598 copies μg of DNA in these specimens (Table 1). Both cases with CMV inclusions were positive by IH with antibody to CMV. The IH stains identified more virus-infected cells in the same biopsy compared to the H&E (Figure 2B).

ISH results were compatible with IH with no additional cases showing positivity (Table 1 and Figure 3). Thrombotic microangiopathy has been associated with CMV. Glomerular thrombi were present in two biopsies (20%) of patients with CMV viraemia. The buffy coat CMV-PCR levels were 10 and 345 viral copies μg of DNA, respectively.

Viral infections may also cause interstitial nephritis. Significant interstitial inflammation (>25% of tissue involved) was seen in 8/13 (62%) of all the biopsies. In five of these, the interstitial inflammation was associated with acute rejection (cases 1, 2, 8–10). The buffy coat CMV-PCR was positive (1976 and 6010 copies μg of DNA) in two of three of those with interstitial inflammation but no rejection (cases 2 and 3, but not case 5).

Glomerular leukocytes have been reported to be present with CMV-glomerulopathy, hyperacute or delayed hyperacute rejection [10]. No patient experienced hyperacute or delayed hyperacute rejection. Glomerular neutrophils were present in 2/13 (15%) biopsies. Only one of these had positive quantitative buffy coat CMV-PCR (276 copies μg of DNA).

CMV disease may proceed, follow, or be seen concomitantly with acute rejection. This can complicate management. Five biopsies (38%) showed acute rejection of Banff grade I-IIA. All five were associated with positive buffy coat CMV-PCR. The quantitative buffy coat CMV-PCR ranged from 34 to 6630 copies μg of DNA. The allograft tissue CMV-PCR was positive in two specimens with acute rejection and both showed CMV inclusions. Paradoxically, the quantitative buffy coat CMV-PCR was relatively low in these two specimens (239 and 598 copies μg of DNA).

One biopsy showed minimal pathologic findings and the CMV-PCR in tissue and blood were negative. All cases with CMV inclusions were positive by IH with antibody to CMV. The stains compared to the H&E identified more virus-infected cells in the same biopsy

Fig. 2. (A) CMV inclusion in case 1 (Table 1), H&E, ×400. (B) IH shows positivity within glomerular cells, with enlarged as well as non-enlarged nuclei IH, ×400.

Fig. 3. ISH for CMV: 1, kidney sections show positive nucleus in an endothelial cell; 2, lung sections with CMV pneumonitis show multifocal positivity within enlarged nuclei of pneumocytes, similar to the kidney signal (positive control).
Table 2. Sensitivity and specificity of blood and tissue CMV-PCR

<table>
<thead>
<tr>
<th>Buffy coat PCR</th>
<th>Tissue positive/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 100</td>
<td>0/5</td>
</tr>
<tr>
<td>&gt; 100</td>
<td>5/8</td>
</tr>
</tbody>
</table>

Less than 100 viral copies in blood effectively rules out invasive CMV infection.

Table 3. Relationship of tissue CMV-PCR positivity to quantitative buffy coat CMV-PCR

<table>
<thead>
<tr>
<th>Tissue PCR</th>
<th>Blood PCR (mean/median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>1834/598</td>
</tr>
<tr>
<td>Negative</td>
<td>998/22</td>
</tr>
</tbody>
</table>

Discussion

Documentation of an association between CMV infection and glomerular disease in the transplanted kidney has been difficult to confirm [11]. Direct evidence of invasive CMV disease with cytomegalic inclusion is rarely found in allograft renal biopsies (<1%), even with an elevated serum creatinine with apparent CMV disease [5]. The reason for this is most likely that the sensitivity of routine histopathology is too low for detection of CMV in renal allograft tissue. Various other histopathologic lesions have been associated with CMV nephropathy and include glomerular leukocytes, glomerular thrombi, interstitial nephritis, acute and chronic rejection [11–16]. These lesions may be seen with other diseases as well. We demonstrated the presence of all of these lesions except chronic rejection in our biopsy specimens. There was no obvious correlation among the histology, buffy coat and tissue CMV as detected by routine histology, IH, ISH and tissue CMV-PCR.

Qualitative and quantitative blood CMV-PCR are currently used to direct therapy in kidney transplant patients [4]. In our previous studies, we have found that a threshold of 500 copies/μg of DNA from blood leucocytes is a reliable indicator of developing symptomatic CMV disease [3]. This suggested that tissue CMV-PCR may be useful to guide therapy for CMV when there is an elevated serum creatinine. In this study, we were able to demonstrate that 50% of patients with CMV viraemia had CMV nephropathy as determined by an elevated serum creatinine and demonstrable allograft tissue CMV infection as detected by PCR. Thus, we were able to demonstrate that renal allograft CMV infection detected by PCR is much more common than previously reported and this has important implications for patient management.

It seems intuitive that a higher quantitative viral load in blood would increase the likelihood of CMV inclusions in renal tissue of patients with CMV disease and nephropathy. The relationship between quantitative blood CMV and CMV renal allograft pathology has not been systematically investigated, however. In this study, <100 copies/μg of DNA (100 000 WBCs) predicted a negative tissue PCR with 100% sensitivity. Qualitative buffy coat CMV-PCR levels, however, did not correlate with detection of CMV inclusions in renal tissue. Paradoxically, quantitative buffy coat CMV-PCR was low (239 and 538 copies/μg of DNA) when CMV inclusions were detected in renal tissue. Whenever tissue is analysed for CMV by PCR, there is always the concern that the PCR may detect CMV in circulating and not resident cells. Our paradoxical finding argues against the possibility that detection of CMV by PCR could simply reflect detection of CMV in circulating leucocytes. The fact that neither of the cases with glomerular leukocytes (cases 11 and 12) had detectable CMV by PCR also mitigates against this possibility. Further, only two of the three cases with interstitial inflammation had detectable CMV by PCR. Remarkably, others have found CMV genomes in renal biopsies even in seronegative patients [7].

The relationship between acute rejection and CMV is complex. Acute rejection may be seen to proceed, follow, or occur simultaneously with CMV. In this study, five patients with biopsy-confirmed acute rejection had CMV viraemia and three of these had allograft CMV infection as detected by PCR.

We conclude that CMV nephropathy is much more common than previously reported when sensitive techniques such as CMV-PCR are used for detection of CMV in tissue. Acute rejection and CMV viraemia occur commonly together in patients at risk for CMV. This has important implications for management of patients who have elevated serum creatinines and are at risk for CMV disease. These findings suggest that dual therapy with anti-rejection and anti-CMV agents are warranted for this group of patients. Finally, although extremely low quantitative buffy coat CMV-PCR levels (<100 copies/μg of DNA) effectively rule
out CMV infection in the renal transplant, the level of quantitative buffy coat CMV-PCR does not correlate with the presence of CMV inclusions on renal allograft biopsy.

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References


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