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Extensive human cytomegalovirus (HCMV) genomic DNA in the renal tubular epithelium early after renal transplantation: relationship with HCMV DNAemia and long term graft function.

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Running head: HCMV in renal biopsies

Keywords: rejection, CMV disease, humoral, in-situ, infection

Abbreviations: ISH, in situ hybridization; IHC, immunohistochemistry, DCT,

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Abstract

Human cytomegalovirus (HCMV) infection is associated with a series of direct and indirect effects following renal transplantation. However, the presence of HCMV in the kidney and its relationship with acute rejection and long-term graft function remain to be fully elucidated. Sixty two biopsies derived from 30 renal transplant recipients with signs of clinical rejection were analysed for HCMV using a sensitive in situ DNA hybridization method. Biopsies were also subjected to staining with anti-C4d antibodies and an anti-caspase 3 antibody to detect humoral rejection and apoptosis respectively. In 21 patients, serial serum creatinine levels over 5 years of follow-up were analysed.

HCMV DNA was detected in biopsies from 21/30 (70%) of the patients and 32/62 (52%) of the individual biopsies. HCMV DNA was detected early after transplant and was localized to renal tubule epithelial cells but not associated with apoptosis. HCMV DNAemia developed within 2 weeks of detecting HCMV DNA in the biopsy in 53% of patients. Ninety percent of patients experiencing HCMV disease had HCMV DNA in their biopsy. HCMV DNA was equally distributed between patients with or without histological evidence of acute rejection and was detected more frequently in patients with peritubular C4d deposits. Creatinine levels at 12-months post transplant were significantly higher in patients with HCMV DNA and remained elevated over the 5 years of follow-up. HCMV DNA is frequently detected in renal tubular epithelial cells early after renal transplantation, precedes DNAemia and is associated with poor long term graft function.
Introduction

Over the last decade, substantial progress has been made in our understanding of the pathogenesis of human cytomegalovirus (HCMV) disease and its clinical management after organ allotransplantation (reviewed by [Fishman et al., 2007]). In addition, the complexity of the virus-host relationship for HCMV continues to unfold [Mocarski Jr et al., 2004; Lilley et al., 2005]. *In vivo*, HCMV replicates rapidly in the immunocompromised host with doubling times as rapid as one day [Emery et al., 1999]. Serial measurements of viral replication in the blood of recipients of renal, liver and cardiothoracic transplants have demonstrated, in the absence of antiviral prophylaxis that HCMV DNAemia occurs frequently with a peak incidence between 30-40 days after transplantation. It was shown previously that high viral loads and replication rate are key factors in the pathogenesis of end-organ disease such as enteritis, hepatitis, retinitis and pneumonitis [Emery et al., 2000] although recent data indicate that extensive periods of lower level replication may also play an important role in the pathogenesis of HCMV [Regoes et al., 2006]. These HCMV diseases are termed collectively the “direct effects” of HCMV. Placebo-controlled trials have shown that after solid organ transplantation these direct effects can be prevented through antiviral prophylaxis with oral ganciclovir [Schmidt et al., 1991; Gane et al., 1997], valaciclovir [Lowance et al., 1999] and more recently valganciclovir [Paya et al., 2004]. Similarly, the direct effects can be minimised using a pre-emptive therapeutic approach [Einsele et al., 1995; Mattes et al., 2004; Humar et al., 2005; Khoury et al., 2006] based upon laboratory markers of replication.
In addition to the direct effects summarized above, HCMV is associated with a variety of other medical conditions including graft rejection, post transplant vasculopathy, post-transplant diabetes mellitus, a generalized immunosuppressive syndrome leading to opportunistic viral and bacterial/fungal/protozoal infections. These effects are termed the "indirect effects" since the virus is not seen histopathologically in the affected organs (reviewed in Fishman et al., 2007). Evidence for a causal role for HCMV in these conditions is based upon clinical observations in individual patients, statistical association between markers of HCMV infection (including IgG seropositivity, asymptomatic viremia and HCMV disease) and the indirect effects in cohort studies as well as from prophylaxis studies [Gratton et al., 1989; Valantine et al., 1999; Lowance et al., 1999]. In particular, the study by Lowance showed that in D+R- renal transplant recipients, biopsy proven acute graft rejection was reduced by 50% in patients receiving high dose valaciclovir. An extensive summary of the impact of anti-HCMV prophylaxis on graft and patient survival after renal transplantation by Opelz [Opelz et al., 2004] adds further weight to the role that HCMV plays in long term graft function and ultimately patient survival. Further evidence has been obtained from the rat CMV models for transplantation where infection is associated with elevated acute and chronic rejection, enhanced inflammatory responses especially up-regulation of VCAM-1, ICAM-1 and LFA-1 [Martelius et al., 1998; Kloover et al., 2000; Martelius et al., 2001]. In addition, rat CMV appears to be present in the transplanted kidney at early times following transplantation but does not persist at high levels despite triggering a series of responses which lead to organ damage [Kloover et al., 2000].
However, not all studies have found an association between acute rejection following solid organ transplantation and HCMV [Boyce et al., 1988; Dickenmann et al., 2001]. Importantly, two other members of the Betaherpesvirinae, HHV-6 and HHV-7 have also been associated with graft dysfunction, organ rejection and HCMV disease [Osman et al., 1996; Ratnamohan et al., 1998; DesJardin et al., 1998; Griffiths et al., 1999; Kidd et al., 2000] and HHV-6 is also sensitive of GCV.

In order to address the question whether HCMV is present in the kidney during the early post transplant period, its relationship with DNAemia and its association with long term organ dysfunction we reasoned that sensitive detection of HCMV DNA was necessary to assess both the quantity and extent of infection. Since conventional methods are unable to provide such sensitivity, we developed a highly sensitive in-situ hybridization methodology based upon riboprobes for HCMV, HHV-6 and HHV-7 DNA which were then used to analyse a series of biopsies obtained from a cohort of renal transplant recipients previously investigated as part of a prospective study of herpesvirus DNAemia [Kidd et al., 2000]. The presence of herpesvirus DNA in the biopsies was then correlated with DNAemia, markers of vascular cellular and humoral rejection, intra-organ cell death and long term graft and patient survival.
Methods

Renal transplant patients

This study was performed on a sub-group of 30 patients from a total of 52 patients enrolled in a previous prospective study who had undergone their first renal transplantation between 1st August 1993 and 31st January 1995 [Kidd et al., 2000]. Surveillance for HCMV DNA in blood was effected once weekly using a quantitative-competitive PCR assay while the patient was in hospital and then at every out patient visit as described in detail in Kidd et al, 2000. Inclusion criteria for the present study was the availability of at least one biopsy, taken when a patient was suspected of having renal dysfunction, during the first 120 days post-transplant. A total of 62 allograft biopsies from 30 renal transplant patients were available taken at a median of 21 days (range 2 to 107 days) after transplantation. Two or more biopsies were available from 18 patients, with a median of 2 (range 1 to 5) biopsies available for the entire cohort. The study was approved by the Local Research Ethics Committee of the Royal Free Hospital. None of the patients received anti-HCMV prophylaxis and all cases of HCMV disease were treated with intravenous ganciclovir (5mg/kg bid adjusted for renal function) for a minimum of 2 weeks or until the clinical signs improved. HCMV disease definition conformed to the criteria of Ljungman et al (2002) which were published after the clinical aspects of this study were undertaken.

Renal biopsies

Biopsy samples were fixed in 4% neutral buffered formalin and paraffin-embedded. Four micron sections were cut and mounted onto
aminopropyltriethoxysilane (APES)-coated slides (Applied Biosystems).

Histopathologic classification of the allograft biopsies followed the Banff working classification.

**Generation of RNA probes for in situ hybridization (ISH)**

PCR was used to amplify specific viral sequences corresponding to the HCMV glycoprotein B gene (UL55; 150bp [Darlington et al., 1991]), HHV-6 U67 (173 bp; [Clark et al., 1996] and HHV-7 U42 (143bp; [Kidd et al., 1996]) genes. The PCR amplicons were then cloned into pGEM-T Easy TA cloning plasmid (Promega). Prior to in vitro transcription, the relevant plasmid DNA construct was linearised with the restriction enzymes Nde 1 or Nco 1 depending upon whether sense or antisense transcripts were required. *In vitro* transcription of the inserted sequences with T7 and Sp6 RNA polymerases (Promega), including incorporation of digoxygenin (DIG)-labelled UTP (Roche) was carried out to generate both labelled sense and antisense ssRNA probes respectively.

**In situ hybridization**

Prior to analyzing the renal biopsy samples, formalin fixed paraffin embedded cells from cultures of HCMV (strain AD169) infected human embryonic lung (HEL) fibroblasts, HHV-6B (strain Z29) infected Molt-3 and HHV-7 (strain DC) infected SupT1 T cell lines were used as positive controls for in-situ hybridization and immunohistochemistry. Non-infected cell cultures were used as negative controls. Formalin-fixed paraffin-embedded human
leukaemic cell line HL60, and HL60 cells treated with 10 µM etoposide, were
used as positive controls for in situ detection of apoptosis.

All pre-treatment solutions were made with distilled water treated with 0.1%
diethylpyrocarbonate (DEPC). Following dewaxing and rehydration, sections
were permeabilised by incubation in 0.2M HCl, followed by 0.01% Triton-X100
in PBS. Sections were treated with 7.5 µg/ml Proteinase K for 30 mins at
37°C and post-fixed with 0.4% paraformaldehyde in PBS. Endogenous
alkaline phosphatase was inactivated by treatment with 20% (v/v) cold acetic
acid. Sections were subsequently washed in water, dehydrated in ethanol,
and air dried. The hybridisation reaction consisted of 50% formamide, 10%
dextran sulphate, I X Denhardt’s solution, 4 X saline-sodium citrate (SSC),
10mM DTT, 1mg/ml denatured and sheared salmon sperm DNA (Sigma), 1
mg/ml yeast tRNA (Roche) and 50 ng of the digoxigenin labelled riboprobe.

After denaturation at 95°C for 5 min, hybridisation was performed at 42°C
overnight. A negative reagent control with hybridization mixture, but without
probe, was performed in parallel for each sample.

Post hybridisation, sections were washed in 4X SSC for 5 min, wash buffer 1
(0.5X SSC, 1 mM EDTA, 50% formamide) at 42°C for 10 min, and 0.5X SSC
at 50°C for 20 mins and equilibration for 5 min at room temperature.

Detection of DIG was carried out using an anti-DIG Fab fragment antibody
conjugated to alkaline phosphatase (Roche) at 1:200 dilution for 2 hours at
room temperature. Development with the chromogen 5-bromo-4-chloro-3-
indoyl phosphate toluidine salt and nitroblue tetrazolium (Vector labs) in 0.1M Tris HCl (pH 9.5) was carried out overnight at room temperature.

Immunohistochemistry

Ten randomly selected renal biopsies which were HCMV DNA positive by ISH were analysed for the presence of HCMV proteins characteristic for each temporal stage of replication and also for evidence of apoptosis using the following monoclonal antibodies (mAbs): mAb8131 (Chemicon, Temecula, CA) directed to the HCMV immediate-early (IE) non-structural Ag of 68-72 kDa; mAb CCH2 (Dako) directed against the delayed-early DNA binding protein of 52 kDa (Plachter et al., 1992); mAb8126 (Chemicon) directed against the late protein of 47-55 kDa; anti-active caspase 3 mAb (C92-605; Phamingen, San Diego, CA) [Dukers et al., 2002]. The specificities of these antibodies were confirmed using isotype matched control mouse mAbs (Dako). Five renal biopsies negative for HCMV DNA by ISH were also included as negative controls. All renal biopsies were tested for the presence of antibody (Ab) mediated rejection using a polyclonal peptide-specific anti-C4d antibody (C4dpAb; Biomedica) [Bohmig et al., 2001]. Normal rabbit IgG serum (Sigma) diluted to the same concentration as the anti-C4d Ab was used as a negative control.

Two immunohistochemical staining methods were used according to the manufacturer’s instructions. The avidin-biotin-peroxidase complex system (Elite ABC kit, Vector Labs) was used with the three HCMV MAbs, and anti-active caspase 3 MAb and the DAKO chemMate Envision detection system
was used with two HCMV MAbs (MAB8131 and MAb CCH2) and with anti-C4d Ab, employing diaminobenzidine as the chromogenic substrate.

Statistical analyses

Statistical comparison between different groups was performed using the $^2$ and Fisher’s exact test or Mann-Whitney test as appropriate. P-values $\leq 0.05$ were regarded as significant.
Results

In situ detection of DNA for HCMV, HHV-6 and HHV-7 in post transplant renal biopsies

Sixty two renal biopsies taken from 30 renal transplant recipients experiencing renal dysfunction who had previously been recruited to a prospective study of betaherpesvirus infection in blood were analysed using the betaherpesvirus specific riboprobes by in situ hybridization. HCMV DNA was detected in 21 of the 30 patients investigated (70%) and in 52% (32/62) of the individual biopsies. HHV-6 and HHV-7 DNA were not detected in any biopsy despite the riboprobe having a comparable sensitivity to the HCMV probe and these patients experiencing HHV-6 and HHV-7 DNAemia in blood (46% and 23% respectively, [Kidd et al, 2000]). HCMV DNA was detected in 8/12 patients with only 1 biopsy available and in a further 13/18 patients where more than one biopsy was available. A significant proportion of the biopsies (30/62) were taken within the first twenty days after transplantation (range 1-19 days) and HCMV DNA was present in 63% of these biopsies. The frequency of detection reduced to 38% (5/13) for biopsies taken between days 20 – 39 and remained relatively static thereafter (42% for biopsies between days 40 and 120 post transplant). In patients who were R+, HCMV DNA was detected in the majority of biopsies (17/23 (74%); D+R+ (11/13 (85%)); D-R+ (5/8 (62%)) which was comparable to in-situ staining in the high risk D+R- group (3/4; 75%). Even in the D-R+ group the transplanted seronegative organ had HCMV DNA present in 5/8 cases during the 120 days of follow-up.
The staining patterns observed for HCMV revealed extensive presence of genomic DNA especially in the epithelial cells of the distal convoluted tubules and proximal convoluted tubules (Figure 1). All the 32 biopsies that were positive for HCMV DNA showed extensive staining in the distal convoluted tubules. The intensity of staining in these cells was commensurate with a relatively high HCMV genome burden. Staining of proximal convoluted tubules, which was less intense than the distal convoluted tubules staining, was evident in 13/32 biopsies as was staining of podocytes. In some biopsies (5/32) weak/moderate staining of endothelial cells and the loop of Henle was also observed.

In order to assess whether the biopsies containing HCMV DNA by in situ hybridization exhibited high level HCMV protein expression or showed evidence of cell death via apoptosis a randomly selected subset of 10 biopsies from different patients were stained for three HCMV antigens (IE-1, p52 and a 47-52Kda late antigen) and with an antibody against the active form of caspase 3. None of the biopsies stained positive for HCMV antigens by immunohistochemistry. In 9 of the 10 biopsies there was no evidence for apoptosis although in 1 biopsy, which was HCMV DNA negative, a small number of apoptotic cells could be observed (data not shown).

**Correlation between HCMV DNA in renal biopsies, HCMV DNAemia in blood and HCMV disease**

The correlation between the presence of HCMV DNA in the renal biopsy and blood is shown in Figure 2. Overall, 75% of the patients with HCMV DNAemia
also had HCMV DNA positive biopsy sample detected in the biopsy. DNAemia occurred within 2–4 weeks after the HCMV DNA positive biopsy in these patients. In 6 patients with HCMV DNA in their biopsy sample, no DNAemia was detected during the 3-months after transplantation.

Ten of the 30 patients included in this study developed HCMV disease. Although disease was more prevalent in the high risk D+R- group (3/4), 42% (5/12) of the D+R+ group and 17% (1/6) of the D-R+ also had HCMV disease including prolonged fever, and gastrointestinal disease. The majority of these patients had HCMV DNA present in their renal biopsy prior to disease onset (9/10; 90%). However, a further 12 of the remaining 20 patients who did not experience HCMV disease also had HCMV present in their biopsy (p = 0.09) illustrating the extensive organ specific infection that occurs in the absence of overt HCMV disease in other organ systems.

**Cellular, vascular and humoral rejection and the presence of HCMV DNA in renal biopsies**

Initially, we investigated whether the presence of HCMV DNA in the renal biopsy was associated with an increased incidence of histologically proven rejection. In samples where acute cellular rejection was observed, 50% (16/32) of the biopsies also contained HCMV DNA whereas in biopsies where there was no rejection or borderline findings HCMV DNA was present in 53% of cases (p = 0.8). A comprehensive summary of the HCMV DNA ISH results in the context of the type of rejection observed histopathologically is shown in Table I.
We also analysed the biopsies for the presence of acute humoral rejection by staining for the complement fragment C4d. Overall, 29/62 biopsies (47%) showed C4d staining in the endothelial cells of the peritubular capillaries (Figure 3). Extensive staining was observed in 10 of these biopsies while in a further 6 (21%) C4d deposits were also observed on the endothelium of the glomeruli. The median time to the detection of C4d was 13 days (range 7 – 64) post transplantation commensurate with the early onset of humoral rejection. The correlation between C4d deposition and histology is summarized in Table 2. In peritubular C4d deposits, acute rejection was more common than in C4d negative biopsies (18/29 vs 14/33) although this did not reach statistical significance (p = 0.1). There was a significantly higher frequency of C4d staining in biopsies classified as Banff type II (vascular) rejection compared to biopsies that were classified as Banff 1 (interstitial) rejection (11/14 vs 5/13; p = 0.03). There was also a strong relationship between C4d staining and intimal arteritis characteristic of vascular rejection (p = 0.005). In the context of HCMV DNA, there was a non-significant trend for HCMV DNA to be found more frequently in biopsies with peritubular C4d deposits than in C4d negative biopsies (18/29 vs 14/33; p = 0.1).

HCMV DNA in renal biopsies and long term outcome

Clinico-pathological details of graft function and patient survival were available for 28 patients (93%). 10-year patient survival was 64% of whom 67% also had HCMV DNA detected in their biopsy by ISH whereas 80% of the deceased patients had a biopsy with HCMV DNA by ISH (p = 0.26).
Functional graft survival over the 10-years of follow-up was 32% (9/28) of whom 55% had HCMV detected by ISH in their biopsy. In contrast, HCMV DNA was detected in 15 of the remaining 19 patients (79%) who had returned to dialysis (prior to death if deceased) although this difference did not reach statistical significance (p = 0.2).

In a subset of patients where full clinical notes were available (n=21) we assessed the impact of HCMV in the renal biopsy and creatinine levels at 3, 12, 24, 36, and 60 months post transplant. The data are shown in Figure 4 and illustrate that the group of patients with HCMV DNA in their biopsy had elevated creatinine levels by 12 months post transplant which persisted during the subsequent 4 years of follow-up. The difference in creatinine levels was statistically significant at 12 months after transplant. Acute rejection episodes were similar between these two groups (50% in patients with HCMV DNA positive biopsies vs 46% in patients without HCMV DNA in their biopsies).
Discussion

In this study we have shown that HCMV DNA can be detected in a high proportion of renal biopsies from early times following renal transplantation. In contrast, HHV-6 and HHV-7 DNA was not present in any biopsy despite some of these patients having blood samples that were positive for these viruses. This observation presumably reflects the source of HHV-6 and HHV-7 is via reactivation of latent virus present in sites outside the transplanted organ. HCMV DNA was observed in the biopsies before the onset of high level DNAemia (~ 20 days) indicating that the local environment in the transplanted kidney provides a suitably rich inflammatory environment to reactivate latent HCMV and allow local replication to occur - the majority of which (~75%) will eventually lead to HCMV DNAemia. The most frequent cells in which HCMV DNA was detected were the distal tubular epithelial cells consistent with the high level excretion of HCMV in the urine of patients following renal transplantation. It is important to note that all the renal biopsies analysed in this study were taken due to renal dysfunction rather than as part of a routine surveillance protocol i.e. patients had clinical evidence of renal malfunction. Interestingly, HCMV DNA was evenly distributed between biopsies that were histopathologically graded as having no/borderline rejection and those with acute rejection. Thus, it is likely that HCMV is a contributor to a clinical picture that resembles rejection of the transplanted kidney although it can also be present without producing pathology associated with classical acute rejection. It is noteworthy that approximately 50% of the biopsies in our study were positive for HCMV DNA and prophylactic studies with valaciclovir or ganciclovir have observed a 50%
reduction in acute rejection in patients receiving the anti-HCMV drugs compared to control groups [Lowance et al., 1999; Ricart et al., 2005]. A criticism of previous studies linking the presence of HCMV DNAemia with acute rejection has been the temporal association of viral replication, and therapeutic intervention to manage the rejection episode i.e. the enhanced immunosuppression deployed may merely promote HCMV replication rather than HCMV itself being intimately involved in the rejection process. In the present study, the treatment for the rejection episode occurred after the biopsy sample was taken and so we believe that our observations at early times after transplantation are consistent with HCMV contributing to the clinical rejection rather than being a consequence of the enhanced immunosuppression given to treat a rejection episode. Nevertheless, due to the extensive presence of HCMV DNA in the tubular epithelium of the transplanted kidney, therapy for acute rejection will facilitate HCMV replication by further reducing local immune effector functions [Gerna et al., 2006].

Although a number of studies have assessed HCMV DNA in renal biopsies [Holma et al., 2000; Liapis et al., 2003], most have not performed the analysis in the context of humoral and cellular rejection and long term graft outcome. We showed that acute antibody mediated rejection is a relatively common finding in renal allografts at a median of 13 days after transplantation and was more likely to be associated with a histological diagnosis of vascular rejection consistent with the recruitment of monocytes to peritubular and glomerular capillaries [Fahim et al., 2007]. We also observed a trend for HCMV DNA to be found more frequently in biopsies with peritubular C4d deposits than in
C4d negative biopsies, the significance of which requires further, more extensive, studies.

HCMV infection in other organ systems has been associated with an increased level of bystander cell apoptosis [Chiou et al., 2001]. HCMV encodes a number of anti-apoptotic genes many of which are expressed at immediate early times during infection that prolong survival of the infected cell (reviewed by [Andoniou et al., 2006]). In the light of these data, we stained the HCMV DNA positive biopsies with an anti active caspase-3 antibody but could detect evidence for apoptosis in only 1 of the 10 biopsies tested. We conclude therefore that HCMV is not associated with increased apoptosis in either infected or bystander cells in the kidney at early stages after transplantation.

Finally, we addressed whether HCMV DNA in the renal biopsy at early times impacted on long term graft and patient survival. Previous analyses of large databases have indicated that anti-HCMV prophylaxis is associated with improved long term graft and patient survival [Opelz et al., 2004]. In our study HCMV DNA was present in the biopsies of 80% of patients who had died by 10-years of follow up, while this figure was 67% in patients still alive. In addition, there were trends for HCMV DNA in the biopsy to be associated with poorer organ function and the necessity for patients to return to dialysis. In a subset of patients with creatinine measurements extending to 5-years post transplant there was a significant elevation in creatinine levels at 12 months in patients who had HCMV DNA in their biopsy and this elevation remained for
up to 5 years and was not confounded by an excess of acute rejection episodes in the group with elevated creatinine levels. Consistent with our data, Lautenschlager and colleagues have also shown a significant association between HCMV DNA in renal biopsies and reduced creatinine clearance at 1 and 2 years post transplantation [Helantera et al., 2006].

In conclusion, we have provided further insight into the indirect effects of HCMV following renal transplantation and show that up to 50% of renal biopsies in patients with renal dysfunction have HCV DNA which precedes the onset of HCMV DNAemia and which in turn is associated with long term renal impairment.
Table I. Association between rejection and presence of HCMV DNA in renal biopsies. The samples were classified following histology as having acute rejection (n=27) or having changes associated with acute rejection (n=37).

<table>
<thead>
<tr>
<th>Histology</th>
<th>All biopsies (%)</th>
<th>HCMV DNA in biopsy</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>Yes (%)</td>
<td>No (%)</td>
</tr>
<tr>
<td>No rejection n (%)</td>
<td>16/62 (28)</td>
<td>7/16 (44)</td>
<td>9/16 (56)</td>
</tr>
<tr>
<td>Borderline rejection n (%)</td>
<td>14/62 (23)</td>
<td>9/14 (64)</td>
<td>5/14 (36)</td>
</tr>
<tr>
<td>Acute rejection n (%)</td>
<td>32/62 (52)</td>
<td>16/32 (50)</td>
<td>16/32 (50)</td>
</tr>
</tbody>
</table>

Acute rejection

<table>
<thead>
<tr>
<th></th>
<th>(%)</th>
<th>Yes (%)</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banff 1 (%)</td>
<td>13/27 (48)</td>
<td>7/13 (54)</td>
<td>6/14 (43)</td>
</tr>
<tr>
<td>Banff 2 (%)</td>
<td>14/27 (52)</td>
<td>6/14 (43)</td>
<td>8/13 (61)</td>
</tr>
<tr>
<td>Glomerulitis (%)</td>
<td>5/32 (14)</td>
<td>1/5 (20)</td>
<td>4/5 (80)</td>
</tr>
</tbody>
</table>

Inflammation
consistent with acute rejection

<table>
<thead>
<tr>
<th></th>
<th>(%)</th>
<th>Yes (%)</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial inflammation n (%)</td>
<td>35/37 (95)</td>
<td>18/35 (51)</td>
<td>17/35 (49)</td>
</tr>
<tr>
<td>Tubulitis n (%)</td>
<td>35/37 (95)</td>
<td>17/35 (49)</td>
<td>18/35 (51)</td>
</tr>
<tr>
<td>Intimal arteritis n (%)</td>
<td>14/37 (38)</td>
<td>6/14 (43)</td>
<td>8/14 (57)</td>
</tr>
</tbody>
</table>
Table II. Association between rejection and presence of C4d in the peritubular cells (PTC) within renal biopsies. The samples were classified following histology as having acute rejection (n=27) or having changes associated with acute rejection (n=37).

<table>
<thead>
<tr>
<th>Histology</th>
<th>All biopsies (%)</th>
<th>C4d in PTC</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes (%)</td>
<td>No (%)</td>
</tr>
<tr>
<td>No rejection n (%)</td>
<td>16/62 (28)</td>
<td>6/16 (38)</td>
<td>10/16 (63)</td>
</tr>
<tr>
<td>Borderline rejection n (%)</td>
<td>14/62 (23)</td>
<td>5/14 (36)</td>
<td>9/14 (64)</td>
</tr>
<tr>
<td>Acute rejection n (%)</td>
<td>32/62 (52)</td>
<td>18/32 (56)</td>
<td>14/32 (44)</td>
</tr>
</tbody>
</table>

**Acute rejection**

| Banff 1 n (%)          | 13/27 (48) | 5/13 (54) | 8/14 (62) | 0.4 |
| Banff 2 n (%)          | 14/27 (52) | 11/14 (43) | 3/13 (21) | 0.02 |
| Glomerulitis n (%)     | 5/32 (14)  | 3/5 (60)  | 2/5 (40)  | 0.6  |

**Inflammation**

**consistent with acute rejection**

| Interstitial inflammation n (%) | 35/37 (95) | 16/35 (46) | 19/35 (54) | 0.13 |
| Tubulitis n (%)               | 35/37 (95) | 16/35 (46) | 19/35 (54) | 0.13 |
| Intimal arteritis n (%)       | 14/37 (38) | 11/14 (79) | 3/14 (21)  | 0.005 |
**Figure Legends**

**Figure 1**

In situ hybridization for HCMV DNA in renal biopsies from 3 patients (Patient 1, panels A & B, Patient 2, D & E, and Patient 3, F) showing extensive staining in renal tubular epithelial cells. A negative control for Patient 1 (no probe) is shown in panel C. Arrows indicate positive nuclear staining for HCMV DNA.

**Figure 2**

Venn diagram showing the relationship in patients between the presence of HCMV DNA in renal biopsy samples and systemic replication of HCMV (DNAemia).

**Figure 3**

Analysis of humoral rejection by detection of C4d deposition in a renal biopsy. Diffuse staining is apparent in endothelial cells in the peritubular capillaries (panel A) and in the glomeruli (panel B). Panel C is the IgG control which shows no background staining.

**Figure 4**

Creatinine levels at 3, 12, 24, 36 and 60 months post transplant in patients with or without HCMV DNA in the biopsy. The median level of creatinine and the 25<sup>th</sup> and 75<sup>th</sup> percentiles are shown. The difference in levels was significant at 12 months.
References


Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. Blood 86:2815-2820.


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Figure 1 Li et al

A

B

C

D

E

F
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HCMV DNA in renal biopsy

HCMV DNA in blood

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