Longitudinal analysis of cytomegalovirus load in renal transplant recipients using a quantitative polymerase chain reaction: correlation with disease

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Serial surveillance samples of urine collected from 103 renal transplant recipients were analysed by polymerase chain reaction (PCR) for the presence of human cytomegalovirus (HCMV) DNA. The PCR results were consistently negative in 70 patients, none of whom developed HCMV disease, and PCR positive in 33 patients of whom 10 developed HCMV disease ($P < 0.001$). In 12 patients, PCR results were positive in three or more consecutive samples indicating extensive HCMV replication. HCMV load in 104 samples from these patients was analysed using a quantitative co-amplification PCR system. The maximal viral burden in the symptomatic patients ranged from $10^6$ to $10^{12}$ genomes/ml urine (median $10^6$) and in the asymptomatic patients from $10^4$ to $10^5$ genomes/ml urine (median $10^5$). The $10^{12}$ difference between these median values was significant ($P < 0.01$). Individual kinetic profiles of viral burden showed that high levels of HCMV correlated with clinically apparent disease. In the majority of the asymptomatic individuals HCMV load remained between $10^4$ and $10^5$ genomes/ml urine; however, in two patients fluctuations in viral load were observed involving higher viral levels (up to $10^{12}$ genomes/ml urine) suggesting that immune responses able to modulate viral replication could be studied in individual patients. Analysis of the temporal appearance and quantity of HCMV in the urine with alterations in white cell numbers showed that leukopenia occurred following the appearance of HCMV in the urine of symptomatic patients but preceded HCMV in the urine of asymptomatic patients ($P = 0.01$). Overall, these results show that longitudinal analysis using fully quantitative PCR methods for HCMV can provide insight into the natural history of HCMV disease in renal transplant recipients.

Introduction

The diseases caused by infection with human cytomegalovirus (HCMV) in immunocompromised patients and particularly in renal transplant recipients are well recognized and documented (Meyers et al., 1990; Balfour et al., 1989; Paya et al., 1989; Pass et al., 1980; Peterson et al., 1980; Rubin et al., 1977; Dummer et al., 1985). These studies clearly show that HCMV has a complex interplay with its host such that particular diseases are frequently observed in specific groups of immunocompromised patients. Thus, HCMV pneumonitis is a frequent manifestation of HCMV infection in the bone marrow recipient, whereas HCMV hepatitis is observed in liver transplant patients and HCMV retinitis in the AIDS patient. HCMV is normally controlled by host T cell immune responses so HCMV disease presents when these cell mediated responses are impaired, either through immunosuppressive drugs or organ transplantation, immaturity in the case of the neonate or through human immunodeficiency virus infection. Potentially, a number of factors may influence the ability of the host to control active HCMV infection and/or disease. These include the degree of immunosuppressive therapy, graft versus host disease, damage to target organs during conditioning of bone marrow transplant recipients, type of transplant performed, and finally the viral burden.

In animal experiments with a variety of viruses, the quantity of virus inoculum is directly related to the severity of subsequent symptoms. Recently, such data have been provided for murine cytomegalovirus infection of mice (Shanley et al., 1993). However, in the context of human medicine, correlation between the quantity of any virus and clinical outcome has, until relatively recently, been difficult to demonstrate formally. One of the earliest reports in this area was in 1975 by C. A. Alford and colleagues (Stagno et al., 1975). In a seminal series of experiments using conventional cell culture methods, the
titre of HCMV present in the urine of congenitally infected infants in the first 3 months of life was shown to be directly related to the severity of HCMV disease ultimately experienced by these infants (Stagno et al., 1975). The sensitivity of cell culture was sufficient to obtain these data since neonates excrete large amounts of HCMV in a relatively small volume of urine. Other groups of immunocompromised patients do not excrete such large amounts of virus and it is usually diluted within a large volume of urine so that quantification of viral load by TCID$_{50}$ determinations has not been routinely performed. In order to obtain such data, more sensitive and quantitative assays must be utilized. At present, there are a number of methods under evaluation for the sensitive detection of HCMV. These include DNA dot-blot hybridization (Saltzman et al., 1992), lymphocyte antigen detection (Van der Bij et al., 1988) and the polymerase chain reaction (PCR; Kidd et al., 1993; Einsele et al., 1991). All these assays can provide qualitative and, if properly adapted, quantitative information. To date, the most sensitive approach for the detection of HCMV in the immunocompromised patient has been PCR.

Several methods for performing quantitative PCR have been reported including endpoint dilution, quantification using external and internal standards and competitive PCR using a target sequence mimetic (reviewed in Fox & Emery, 1992). The latter method is proving to be the most accurate method for quantification and has been applied to many viral systems including HCMV (Fox et al., 1992; Schafer et al., 1993). The HCMV method previously reported by our laboratory (Fox et al., 1992) utilizes a control sequence within each PCR that is identical to the target sequence except for the modification of two base pairs to yield a restriction site for HpaI. Following amplification, digestion of the products and electrophoresis resolves the control amplicon from the target amplicon. Comparison of the areas of the resolved products by scanning densitometry allows the original amount of target HCMV DNA present in the specimen to be calculated. We have shown that such a method has a broad dynamic range and that the ratio of amplifiable HCMV genomes to HCMV particles that are infectious in human embryo lung fibroblast cell culture is 2000:1 (Fox et al., 1992).

On the basis of these observations, we were interested in applying the quantitative PCR methods to investigate alterations in the HCMV load in the urine of immunocompromised patients and to correlate changes in viral burden with appearance of disease markers, immune status and antiviral chemotherapeutic intervention. Any differences observed between patients with and without disease could then be correlated with the data already available for HCMV infection (Saltzman et al., 1992; Stagno et al., 1975; Landry & Ferguson, 1993) and provide insight into HCMV pathogenesis. On the basis of the differing patterns of HCMV infection and disease in particular patient groups (vide supra) we reasoned that such analysis should be performed on a single patient group. Since we have previously undertaken extensive prospective analyses of HCMV infection and disease in the renal transplant group (Grundy et al., 1987, 1988; Iragorri et al., 1993; Lui et al., 1987; Pillay et al., 1993), we have applied the quantitative PCR methodology to investigate fluctuations of viral load in the urine of these patients.

Methods

Study population. At our Institution, we receive routine surveillance samples of blood and urine from patients post-renal transplantation. These are collected weekly while the patient remains in hospital and at every out-patient clinic thereafter (Pillay et al., 1992, 1993; Grundy et al., 1987; Lui et al., 1987; Iragorri et al., 1993).

HCMV disease. HCMV disease was defined as clinical features typical of HCMV occurring within 2 weeks of laboratory-documented HCMV infection in the absence of other diagnoses. HCMV disease was classified according to the system described by Plotkin et al. (1991). The disease patterns observed for the six symptomatic patients were as follows. Patient 1: pyrexia (26 days), shortness of breath at the time of pyrexial onset and HCMV pneumonitis. Patient 2: acute pyrexia (15 days duration) followed by HCMV colitis. Patient 3: acute pyrexial debilitating disease (6 days duration), tachycardia. HCMV hepatitis as assessed by raised liver function tests and HCMV duodenitis. Patient 4: febrile for 10 days. Patient 5: pyrexia (10 days duration) and diarrhoea. Patient 6: acute pyrexia (10 days) followed by pyrexia and cough. In the cases of HCMV organ disease, HCMV presence was confirmed by histology. Patient 1 was prescribed a course of ganciclovir and patient 3 was prescribed a course of acyclovir.

There were 21 patients who were HCMV PCR positive in urine on less than three consecutive occasions who were not subjected to quantification of HCMV load. The patients comprised four who were assessed, as described above, to have symptomatic infection with HCMV. Three of these patients had mild pyrexia whilst the fourth patient had HCMV retinitis requiring treatment with ganciclovir and HCMV immune-globulin.

Sample preparation. In the current study, 510 urine samples from 103 individual renal transplant recipients were tested by qualitative PCR. The number of samples available from each patient ranged from three to fifteen. Samples from the patients found to be consistently PCR-positive (i.e. to have three or more consecutive positive samples) were analysed in the subsequent quantitative PCR studies, using the method described previously (Fox et al., 1992). Both the initial qualitative PCR study and the quantitative PCR evaluation were performed blind, i.e. the disease status of the individual patients was unknown to the laboratory investigators.

The samples used in these studies were stored as frozen aliquots at −70 °C. Following thawing at room temperature, urine samples were used directly as PCR target material. Neat urine (2 μl) was added to 98 μl of the PCR reaction mix, a cocktail containing appropriate concentrations of PCR reagents as described below (Fox et al., 1992; Darlington et al., 1991; Kidd et al., 1993).

Polymerase chain reaction. The sequences of the primers used for PCR were 5' GAGGACAACGAAATCCTGTGGGCA (GB1)
and 5' TCGACGGTGGAGATGCTGAGG (GB2); anneal to positions 81683–81707 and 81559–81582 respectively in the prototype AD169 sequence (Chee et al., 1989). The amplified product was a 149 bp fragment of DNA (Darlington et al., 1991). The conditions for PCR were as follows: buffer (1x), 25 mM-Tris-HCl pH 8.4, 17 mM-ammonium sulphate, 2 mM-magnesium chloride, 0.1% gelatin and 0.5 mM-beta-mercaptoethanol, 200 μM of each deoxynucleotide triphosphate, 5 units of Taq DNA polymerase (Applied Biosystems) and 170 μM of each primer. Cycling conditions consisted of 94 °C for 4 min followed by 35 cycles of 60 °C for 30 s, 72 °C for 30 s and 94 °C for 30 s. The final cycle consisted of an extension reaction at 72 °C for 10 min. Initial qualitative PCR screening was carried out in duplicate on undiluted and a 1/20 dilution of the sample and if discrepant results were obtained the sample was re-tested both neat and at a 1/20 dilution (Kidd et al., 1993). If neither of the repeat reactions gave a band of the expected size (149 bp), the sample was classified as negative. If, however, one or both of the repeat reactions gave a band of the expected size, the sample was classified as HCMV positive. All samples from any patient that exhibited three or more consecutive positive reactions by qualitative PCR for HCMV were subjected to quantitative PCR using the method described in Fox et al. (1992). Briefly, 1000 copies of a control sequence that was identical to the target sequence except for a 2 bp mutation to yield at a HpaI restriction endonuclease site was added to each PCR. In the quantitative method the PCR was performed using a 5' 32P-labelled GB1 primer. Following PCR, the products of the reaction were phenol extracted and precipitated with ethanol and the resulting DNA pellet was resuspended in water (20 μl). An aliquot (10 μl) of the product was digested with HpaI in a total volume of 20 μl using established methods (Sambrook et al., 1989). The digested products were mixed with glycerol dye mix (5 μl) and electrophoresed through a 12% polyacrylamide gel in a minigel apparatus (ATTO) at 150 V for 30 min. Following electrophoresis, the gel was covered in clingfilm and exposed to Hyperfilm MP (Amersham) for 12 h. The signals due to target and control amplimers were quantified by scanning densitometry of the autoradiograph using an ACD 2010 densitometer (Gelman). The ratio of the signals due to target and control amplimers was then used to calculate the number of genomes present in the original clinical sample.

The quantitative PCR assessment of positive samples was carried out in triplicate at either neat or 1/20 dilutions of the urine as appropriate. The quantitative PCR assay had an inter-assay reproducibility on clinical samples of approximately 5% and an intra-assay reproducibility of 3.5%. These data were derived by analysis of three different patient samples containing approximately 100 genomes, 1000...
genomes and 10 000 genomes respectively in triplicate in the same assay or in different assays. The results obtained for the 100 and 10 000 genomes were normalized to 1000 genomes and the mean and standard deviation of each data set calculated. The inter- and intra-assay reproducibility represent the mean standard deviation expressed as a percentage.

In order to exclude the possibility of PCR contamination by either cloned target or HCMV-containing sample material the recommendations of Kwok & Higuchi (1989) were adopted throughout.

Statistical analysis. The significance of the results was determined using Fisher's exact test or the Mann Whitney U test as appropriate.

Results
A total of 103 renal transplant recipients were investigated for the presence of HCMV in sequential urine samples by PCR. Of these 103 patients, 70 remained qualitatively PCR negative throughout the period of surveillance and none of these patients had evidence of disease. However, 10 of the remaining 33 patients who were qualitatively HCMV PCR positive suffered HCMV disease (P < 0.001). This stratification is summarized in Fig. 1. In order to gain insight into the fluctuations of HCMV load in patients who were either symptomatic or asymptomatic for HCMV disease we selected 12 patients for further analysis on the basis of availability of three or more consecutive HCMV PCR positive samples. Six of these patients were symptomatic and six had no evidence of disease during the surveillance period (Fig. 1). The number of urine samples quantified on the six asymptomatic patients was 51 and on the six symptomatic patients was 53. The characteristics of these patients with

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<th>Table 1. Patient details*</th>
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* Including sex, age at time of transplant, reason for transplant, HCMV sero-status of donor and recipient, graft rejection episodes and graft survival for the six asymptomatic patients and six symptomatic patients for whom quantitative PCR was performed. Median and ranges of creatinine levels and urine volumes post-transplant are also shown for each patient. Key: D, donor; R, recipient; IDDM, insulin dependent diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus.
Cytomegalovirus load in renal patients

Day 143 (Target/control = 0) 7.5 × 10^4 genomes/ml

Day 63 (Target/control = 0.15) 7.5 × 10^5 genomes/ml

Day 55 (Target/control = 0.35) 1.75 × 10^6 genomes/ml

Day 37 (Target/control = 2.4) 1.2 × 10^6 genomes/ml

Day 30 (Target/control = 1.15) 5.75 × 10^5 genomes/ml

Day 20 (Target/control = 0.08) 4 × 10^5 genomes/ml

Day 10 (Target/control = 0)

Fig. 2. Sequential stacked densitometric scans obtained from one of the symptomatic patients (patient 3). The signals due to the target and control amplimers are shown at days 10, 20, 30, 37, 55, 63 and 143 post-transplant. The ratio of the target:control amplimers was then used to calculate the number of genomes present in the original urine sample. In this case 1000 copies of the control sequence were used and a 500-fold dilution of the urine analysed.

respect to age, sex, serological status of donor and recipient for HCMV, episodes of rejection and graft survival are summarized in Table 1. Although high creatinine levels were frequently detected immediately following transplantation, the creatinine levels of all patients under analysis did not reach a level that was greater than fourfold higher than the pre-transplant creatinine level during the period when HCMV was also present (a pathological manifestation attributable to HCMV). In addition, median urine volumes did not differ markedly between the two groups of patients although variations within individuals were observed. In the majority of cases fluctuations were greatest immediately post-transplant and urine volumes varied by less than twofold during the period when HCMV was being excreted. All patients, except symptomatic patient 5 who died, remain healthy to date with minimal immunosuppression.

The results from scanning densitometry of PCR products obtained after using the quantitative method in one of the patients under investigation are shown in Fig. 2. Various facets of this figure should be noted. Firstly, it is apparent that the relative distribution of the signal between the target and control species is dependent upon the concentration of the target sequence present. Second, the ratio of target to control amplimers increased approximately 26-fold from day 20 (target:control = 0.08) to day 37 (target:control = 2.1) and then decreased over the subsequent 26 days. When the ratios are converted into genomes/ml urine, the maximum level of viruria on day 37 equates to 10^61 genomes/ml. By performing similar analysis on each of the 12 patients under investigation the fluctuations in HCMV load were evaluated.

The results of these analyses (Figs 3 and 4) for the six symptomatic and six asymptomatic patients show that disease correlated with periods in which high levels of HCMV genomes were detected in the urine. In the majority of cases, the kinetics of HCMV burden follow a single or multiple Gaussian curve. Peaks rise from baseline or are superimposed on a low level of virus load; for example compare the profile of patient 2 with patient 3 (Fig. 3). In the two patients in whom antiviral chemotherapy was initiated (patients 1 and 3, Fig. 3), ganciclovir was associated with a reduction of the viral load by 6 logs over a period of 25 days subsequent to initiation whilst acyclovir was associated with a 10^14 reduction in viral load over 14 days of treatment.

The profiles of HCMV load in the six asymptomatic patients are shown in Fig. 4. Two different patterns of viruria were observed. The first is exemplified by patients 3, 5 and 6 in whom a relatively constant viral burden between 10^4 and 10^5 genomes/ml was detected over time. The second pattern observed for patients 1, 2 and to a lesser extent 4 comprised rapid modulations of viral load over time frequently involving higher levels of HCMV.
Fig. 3. Variation in HCMV genome levels over time in urine from patients who were symptomatic during the period of surveillance. The HCMV load is expressed as $\log_{10}$ genomes/ml urine. Disease type and duration is displayed together with antiviral therapy.

Fig. 4. Variation in HCMV genome levels over time in urine from patients who remained asymptomatic during the period of surveillance. The HCMV load is expressed as $\log_{10}$ genomes/ml urine.
HCMV burden and white blood cell (WBC) levels

The temporal association between viral burden and white cell levels was investigated. The data obtained for all patients analysed by quantitative PCR is shown in Figs 5 and 6. Of the 12 patients analysed, 10 exhibited leukopenia ($<4 \times 10^9$ WBC/l). In all the symptomatic patients HCMV DNA was detected before the leukopenia whereas in the four asymptomatic patients in whom leukopenia was also observed, HCMV DNA was detected following the onset of leukopenia ($P = 0.01$). In 9/10 of the leukopenic patients, HCMV was present during the periods of leukopenia although there was no simple relationship between viral load and the severity or duration of leukopenia. A variety of patterns was observed in WBC numbers with reductions to below $4 \times 10^9$/l appearing either transiently, as seen in symp-
Viral burden and disease

The different kinetics of viral load observed for the 12 patients analysed were used to correlate maximum levels of genomes present in the urine with the disease status of each patient. The results of these analyses are shown in Fig. 7. The median viral burden for the asymptomatic group was $10^{5.2}$ genomes/ml urine whereas the symptomatic group had a median viral burden of $10^{6.5}$ genomes/ml. The difference between the median viral load in the groups was statistically significant ($P < 0.01$) and corresponded to $10^{13}$ genomes/ml.

Discussion

We have used a fully quantitative PCR for HCMV previously developed in our laboratory to analyse fluctuations in HCMV burden over time in the urine of
renal transplant recipients. In order to enable meaningful longitudinal analysis to be effected, we selected patients from a total study population of 103 renal recipients in whom three or more consecutive HCMV PCR results were positive. Whilst this procedure excludes 21 patients who excreted HCMV from the quantitative analysis, we reasoned that quantification at single time-points would be unlikely to provide insight into HCMV pathogenesis.

The results show that substantial variations in HCMV load are apparent both between patients and within an individual patient. The maximum viral burden in patients with symptomatic HCMV infection ranged from $10^{5.9}$ to $10^{7.15}$ genomes/ml urine, whereas the asymptomatic patients had virus loads which were significantly lower ranging from $10^6$ to $10^{5.1}$ genomes/ml urine. The median values of the maximum virus burden detected in the urine of the asymptomatic versus symptomatic patients were separated by $10^{1.8}$ genomes/ml urine. Interestingly, this value is comparable to the difference between urine levels of HCMV in symptomatic and asymptomatic infants congenitally infected with the virus ($1.2 \log_{10}$; Stagno et al., 1975). On the basis of our previous results from two cases of congenital infection showing that the ratio of infectious HCMV in human embryo lung fibroblasts to HCMV genomes is 2000:1 (Fox et al., 1992; unpublished data), the results presented here would correspond to a range of 5 TCID$_{50}$/ml to 7500 TCID$_{50}$/ml. These values are substantially lower than the titres of virus observed in symptomatic congenitally infected infants in which $10^8$ TCID$_{50}$/ml is not uncommon (Stagno et al., 1975) and indicate the potential of quantitative PCR to address the impact of viral load on type and severity of disease in the immunocompromised host. An important consideration in the context of HCMV pathogenesis in specific patient groups is the exact time during the period of surveillance that the maximal viral load is detected and how this parameter relates to the disease process and pathology. In the group of symptomatic patients which we have investigated in detail, high levels of HCMV genome load in urine usually corresponded with the period of illness.

Leukopenia is often a manifestation of HCMV disease (Plotkin et al., 1991) although the temporal appearance of HCMV in the urine or blood and reductions in WBC numbers has not been extensively investigated. The data in the current study support the observation that periods of leukopenia are observed when active HCMV replication is on-going but suggest that in the asymptomatic patients, HCMV in the urine preceded the period of leukopenia whereas in the symptomatic patients, HCMV in the urine succeeded the onset of leukopenia. Although this difference reached statistical significance ($P = 0.01$), more data are required to substantiate this observation and to investigate the inter-relationship between viral load and duration of leukopenia.

Clearly, more longitudinal data on larger numbers of patients will be required before the complete distribution of cytomegaloviral loads in the urine of asymptomatic and symptomatic renal transplant patients and the influence of other confounding variables such as duration of quantity of immunosuppression on viral load can be assessed. However, in the current study, statistically significant differences in viral load between the asymptomatic and symptomatic patients were observed. Other studies have used similar and alternative methods for quantification of HCMV including semi-quantitative PCR (Cagle et al., 1992), quantitative dot-blot hybridization (Saltzman et al., 1992), quantitative antigenemia (Landry & Ferguson, 1993) and competitive nested PCR (Gerdes et al., 1993; Schafer et al., 1993). In the study of Saltzman et al. (1992), AIDS patients and solid organ recipients with visceral HCMV disease had higher levels of genomes in the blood than similar patients without visceral involvement. In the study by Schafer et al. (1993), blood from renal transplant patients was analysed and in three patients with symptomatic infection from a total study group of 17, HCMV load in the blood was approximately $1 \log_{10}$ higher than in patients who remained asymptomatic. Taken together, the results described here coupled with those from other workers (vide supra) clearly demonstrate that HCMV load is an important factor in HCMV pathogenesis and disease. The importance of viral load in pathogenesis has also been shown for hepatitis B and C viruses, human immunodeficiency virus and Lassa fever virus (Connor et al., 1993; Fong et al., 1994; McCormick et al., 1986;
Piatak et al., 1993; Hagiwara et al., 1993; Kato et al., 1993).

In conclusion, our results show that longitudinal studies using quantitative PCR methods can provide insight into many aspects of HCMV infection in renal transplant recipients. These include the relationship between HCMV load in the urine with HCMV disease, temporal association between viral burden and immune competence and the potential to use quantitative PCR to provide information on the response of HCMV to antiviral therapy. Further analyses are required to delineate the dynamics of viral load in the urine, blood and other body sites with disease in other immunocompromised groups in which HCMV pathogenesis differs and to monitor modulations in viral load and disease patterns that result from primary infection, reactivation of latent HCMV and reinfection. These investigations are in progress in our laboratory.

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References


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