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Quantity of Human Cytomegalovirus (CMV) DNAemia as a Risk Factor for CMV Disease in Renal Allograft Recipients: Relationship with Donor/Recipient CMV Serostatus, Receipt of Augmented Methylprednisolone and Antithymocyte Globulin (ATG)

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A prospective longitudinal study of 87 renal allograft recipients identified 31 patients with cytomegalovirus (CMV) viraemia. Previous studies have identified CMV viraemia, donor positivity, and CMV load in urine as independent risk factors for disease following renal transpl antation. We used quantitative-competitive polymerase chain reaction (QC-PCR) to quantify the CMV DNA load in blood from these patients, and report that it is a significant and independent risk factor for CMV disease. Patients with symptomatic CMV infection had significantly higher maximum CMV loads than those with no disease (P = .0003). We also found that peak loads were significantly higher in individuals experiencing primary CMV infection (P < .01), and CMV re-infection (P < .05) compared with recipients reactivating endogenous CMV. Univariate analysis revealed that CMV DNA load in blood, donor seropositivity, and receipt of antithymocyte globulin (ATG) were all significantly associated with disease (P = .005, .04, and .05, respectively). However, the association of donor/recipient serostatus, and receipt of ATG became nonsignificant in multivariate analyses whereas the significance of the quantity of CMV DNAemia was maintained, illustrating that CMV load plays a central role in the pathogenesis of CMV disease. ***J. Med. Virol. 58:182–187, 1999.***

KEY WORDS: cmv load; cmv DNAemia; quantitative-competitive polymerase chain reaction; cytomegalovirus; antithymocyte globulin; serostatus

INTRODUCTION

Human cytomegalovirus (CMV) infections are typically asymptomatic in healthy individuals. However, this virus is now regarded as a major cause of morbidity and mortality in immunocompromised patients such as human immunodeficiency virus (HIV)-infected individuals, immunosuppressed transplant recipients, and in the immunologically immature fetus and infant [Britt and Alford, 1996; Griffiths and Emery, 1997].

Previous studies have shown that up to 50% of renal transplant patients excrete CMV, and between 30% and 40% of these will develop CMV disease [Betts et al., 1977; Balfour et al., 1989]. The importance of CMV load in pathogenesis was originally illustrated by Stagno et al. [1975] who showed that high CMV titres in the urine of congenitally infected infants were significantly associated with disease. More recently, Cope et al. [1997a] showed that the quantity of CMV viruria, measured using a quantitative-competitive polymerase chain reaction assay (QC-PCR; Fox et al., 1992, 1995], is a major risk factor for CMV disease following renal transplantation. Others have shown that the quantity of CMV in peripheral blood cells of HIV-infected indi-

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viduals is an indicator of CMV retinitis [Rasmussen et al., 1995]. Recent findings from our laboratory have shown that elevated levels of CMV DNAemia in liver and bone marrow transplant recipients and HIV-infected individuals is a significant and independent risk factor for CMV disease [Bowen et al., 1996; Cope et al., 1997b; Gor et al., 1998]. Collectively, these studies indicate that CMV viral load is a reliable indicator of the probability of disease.

In addition to their standard immunosuppressive therapy [Irragorri et al., 1993], renal transplant patients are given methylprednisolone and antithymocyte globulin (ATG) to treat graft rejection post-transplant. Augmented immunosuppression has also been associated with an increased risk of CMV disease in renal transplant recipients [Pillay et al., 1993]. Interestingly, the administration of methylprednisolone has been shown to predispose liver transplant recipients to CMV disease at lower CMV DNA levels in patients with CMV viraemia than otherwise would be the case [Cope et al., 1997b].

Using QC-PCR, we have performed a longitudinal study on blood samples obtained from 31 renal allograft recipients to quantify the risk associated with the level of CMV DNAemia and its relationship with donor/recipient (D/R) CMV serostatus, methylprednisolone, and ATG as risk factors for the development of CMV disease. A study of this kind has not been conducted previously in this patient group, and in addition enables the natural history of CMV infections to be investigated, because renal transplant patients at this hospital do not receive antiviral prophylaxis.

MATERIALS AND METHODS

Study Population

A prospective study for the detection of CMV viraemia by qualitative PCR was carried out on 87 renal allograft recipients who had been transplanted between August 1993 and February 1997. A total of 369 surveillance samples were received, and the median number of samples for each patient was 11 (range: 5–25). Fifty-four of these patients were CMV PCR negative in all consecutive samples tested and two were excluded due to the unavailability of complete medical history. The remaining 31 patients had at least one CMV PCR positive result and were subjected to CMV viral load measurement in whole blood. The median number of positive samples for each patient was three (range: 1–14). The median age of each patient on day of transplant was 41 years (range: 13–70 years).

Immunosuppressive Therapy

The standard immunosuppressive regimen followed by renal allograft recipients at the Royal Free Hospital, London has been described previously [Irragori et al., 1993; Pillay et al., 1993; Cope et al., 1997a]. To summarise, all patients were given daily doses of prednisolone to a maximum of 0.3 mg/kg (initially intravenously then oral). Azathioprine was prescribed 24 hr post-transplant at approximately 1 mg/kg per day and this dosage was maintained unless the patient developed neutropenia. Cyclosporin A (whole blood levels by high performance liquid chromatography: 100–175 ng/ml) or FK506 (whole blood levels: 5–15 ng/ml) were also administered post-transplant and this dosage was continued as part of a maintenance therapy. In general, histologically identified rejection episodes were treated with 500 mg/m^2 per day of methylprednisolone for 3 consecutive days. If rejection continued, ATG (Merrieux rabbit ATG) was prescribed for 10–15 days (5 mg/kg per day). The total methylprednisolone and ATG received by this transplant group ranged from 0 to 9,000 mg and 0 to 6,150 mg, respectively.

DNA Extraction and QC-PCR

DNA was extracted from 200 μl of whole blood using a commercially available kit (Qiagen, Germany) and eluted with 200 μl of supplied buffer. Subsequent qualitative and quantitative-competitive PCR amplifications for the detection and quantification of CMV viraemia were performed as described previously but with slight modifications [Fox et al., 1992]. To summarise our QC-PCR assay, 5 μl of extracted DNA were added to a standard PCR reaction mixture containing 1× PCR Buffer II, 2 mM $MgCl_2$, 100 ng each of sense (gB1 (81683–81707): 5′-GAGGACAACGAAATCCTGTTGGGCA-3′) and antisense (gB2 (81580–81558): 5′-GTCGACGGTGGAGATACTGCTGAGG-3′) CMV gB primers, and 1 unit of Amplitaq Gold (Perkin Elmer, Warrington, UK). In each reaction an internal control sequence of known copy number was included. This control sequence, with the exception of 2 bp, is identical in length and sequence to the viral gene product being amplified. Site-directed mutagenesis was used to create a Hpa I restriction site, therefore enabling it to be distinguished from the target DNA. An initial denaturation at 95°C for 12 min was followed by 94°C (30 sec) denaturing, 60°C (30 sec) annealing, and 72°C (30 sec) extension for 40 cycles, with a final extension at 72°C for 10 min. Following PCR amplification, samples were Hpa I digested and separated on a 12% acrylamide gel. Gels were then stained in ethidium bromide (0.15 μg/ml) for 5 min and CMV DNA load/ml of whole blood was calculated by comparing the intensities of the target and control bands using the NIH image analysis program.

CMV Disease and Antiviral Chemotherapy

Patients that met the case definition of CMV disease were diagnosed according to international criteria [Ljungman and Plotkin, 1995]. Pyrexia of 38°C or higher for a minimum of 48 hr with neutropenia and CMV viraemia, but with no evidence of graft rejection or bacterial or fungal infections, was also included as evidence of CMV disease, so-called CMV syndrome.

Antivirals were not prescribed as prophylaxis or preemptive therapy but acyclovir and ganciclovir were administered for herpes simplex virus lesions and CMV disease, respectively, when indicated clinically, according to established dosage regimens.

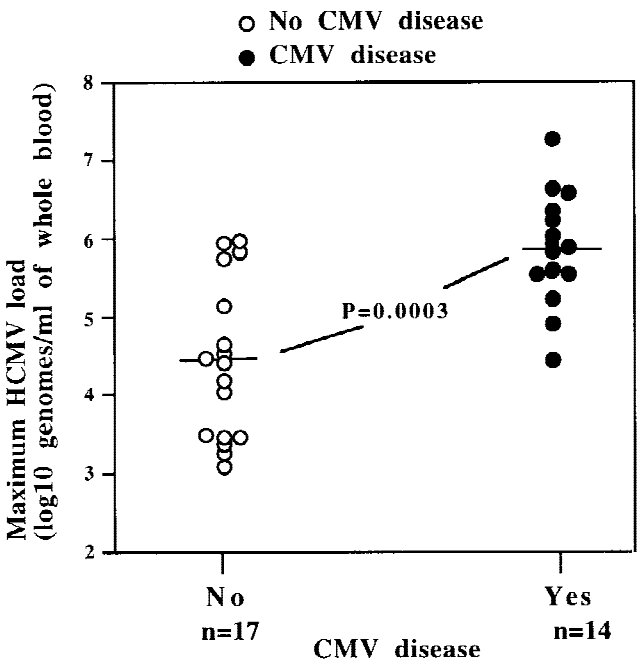

Fig. 1. Scatter diagram showing the relationship between maximum cytomegalovirus (CMV) load in the blood from renal allograft recipients and CMV disease.

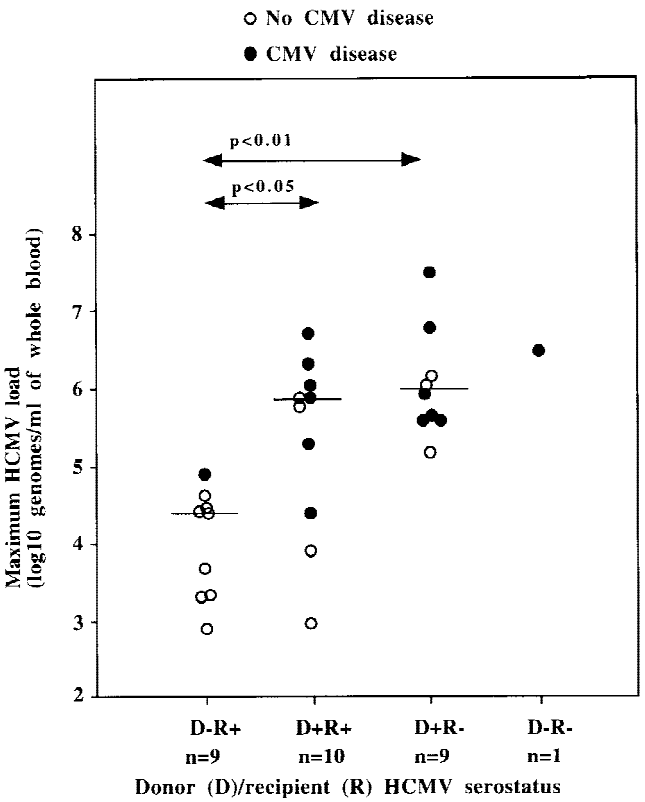

Fig. 2. Scatter diagram showing the relationship between maximum cytomegalovirus (CMV) load in the blood from renal allograft recipients and donor/recipient CMV serostatus.

Statistical Analyses

The differences between the median maximum viral loads in patients with and without CMV disease were assessed by Wilcoxon's test. The Mann–Whitney test was used to observe the significance of differences between the median maximum viral load in the different D/R subgroups. Fisher's exact and Chi-squared tests were applied to assess the relationship between donor CMV serostatus, recipient CMV serostatus, receipt of methylprednisolone, and receipt of ATG with likelihood of CMV disease. Logistic regression analyses (uni- and bivariate) were employed where relationships were significant [Altman, 1993]. Odds ratios (OR) quoted for these analyses are per 0.25 $\log_{10}$ increase in viral load.

RESULTS

A prospective study of 87 renal allograft recipients identified 31 patients with CMV viraemia, of whom 14 (45.1%) met the case definition of CMV disease. The maximum viral load during the post-transplant period ranged from $10^{2.89}$ to $10^{7.51}$ genomes/ml whole blood (median load, $10^{5.26}$ genomes/ml). Peak CMV loads from symptomatic patients ranged from $10^{4.38}$ to $10^{7.51}$ genomes/ml (median peak load, $10^{5.94}$ genomes/ml) and from asymptomatic recipients ranged from $10^{2.89}$ to $10^{6.08}$ (median peak load, $10^{4.36}$ genomes/ml). Thus, patients with CMV disease had significantly higher peak CMV loads compared with those who remained asymptomatic ($P < .0003$, Wilcoxon test; Fig. 1).

Donor CMV serostatus was known for 29 of the 31 patients, 19 (65.5%) of whom were seropositive. Twenty-one of the recipients (67.7%) were CMV seropositive. Twelve of the recipients who received kidneys from CMV seropositive donors had disease, and a significant relationship was observed between donor serostatus and disease ($P = .05$, Fisher's exact test). A significant relationship was also observed between recipient serostatus and absence of disease: only 6 of the 21 seropositive recipients developed disease compared with 7 of 10 seronegative recipients ($P = .05$, Fisher's exact test).

Stratification of peak CMV loads according to D/R CMV serostatus (Fig. 2), illustrated that patients within the D–R+ subgroup (i.e., experiencing CMV reactivation) had significantly lower peak CMV loads (median peak load $10^{4.36}$ genomes/ml) than those in the D+R– and D+R+ subgroup ($P < .01$ and $P < .05$ respectively, Mann–Whitney test). The median peak loads of the latter two groups were similar ($P > .05$). With the exception of one individual, all cases of CMV disease occurred in the D+R+ and D+R– subgroups. Patients in the D+R+ group could have been experiencing either CMV reinfection with a strain of donor virus, or CMV reactivation. There were 7 patients in the D+R+ group with viral loads above $10^{5.0}$ genomes/ml, 5 of whom (71.4%) had CMV disease. In total, there were 14 patients with CMV loads less than $10^{5.0}$ genomes/ml; however, complete D/R serostatus was known for 12 of these patients. Of the 14, two had CMV disease and 3

were from the D+R+ subgroup. Viral loads less than $10^{5.0}$ genomes/ml were comparable to the CMV loads observed for the D–R+ group, and patients were therefore likely to be experiencing reactivation of latent virus. There were 3 patients in the D+R+ group who had CMV loads less than $10^{5.0}$ genomes/ml.

Of the 31 patients, there were 6 patients who received neither methylprednisolone nor ATG and only 1 developed CMV disease. This patient was within the D+R– CMV serostatus group, and had a high level of virus (peak load, $10^{5.98}$ genomes/ml). Fifteen patients received ATG (range: 10–6,150 mg; median, 1,500 mg) and 23 received methylprednisolone (range: 625–9,000 mg; median, 4,500 mg). Patients receiving ATG exhibited a higher peak viral load compared with those who were not treated, whereas receipt of methylprednisolone was associated with a lower peak viral load (Fig. 3). However, these differences did not did not reach statistical significance.

Table I shows the results from the univariate and multivariate logistic regression analyses. Univariate analysis revealed that CMV load (OR 1.66/0.25 $\log_{10}$ increase) was a highly significant risk factor for CMV disease ($P = .005$), together with receipt of ATG (OR 4.50, $P = .05$), whereas recipient seropositivity was associated with a significantly lower risk of CMV disease (OR 0.17, $P = .04$). There was a nonsignificant trend for donor seropositivity to be a risk factor for CMV disease (OR 5.5, $P = .06$). In bivariate logistic regression analyses, CMV load (per 0.25 $\log_{10}$ increase) remained a significant risk factor for disease after controlling for D/R CMV serostatus, or receipt of ATG. In contrast, the significance of the lower risk associated with recipient seropositivity, and the increased risk for CMV disease associated with receipt of ATG were negated once viral load had been controlled for.

The sensitivity and specificity of the QC-PCR assay at various viral load cut-off values is shown in Table II. The assay had maximum sensitivity at viral load cut-off values from $10^{3.5}$ to $10^{4.5}$ genomes/ml, and the specificity of the assay increased by 6% and 22% with each 0.5 $\log_{10}$ increase in viral load to a maximum specificity of 67% when sensitivity was maximal. A further 0.5 $\log_{10}$ increase in viral load resulted in an 8% decrease in sensitivity but a 5% increase in specificity, and a 1.0 $\log_{10}$ ($10^{5.5}$ genomes/ml) decreased the sensitivity to 85% but increased the specificity to 78%. However, a further 0.5 $\log_{10}$ resulted in a 39% decrease in sensitivity, although the specificity of assay increased to 89%. This analysis suggests that the assay had optimal sensitivity and specificity at viral load strata between $10^{4.5}$ to $10^{5.5}$ genomes/ml.

DISCUSSION

The data obtained from this study illustrate that the quantity of CMV viraemia in renal allograft recipients is a significant risk factor for CMV disease. Figure 1 shows that patients symptomatic with CMV disease had significantly higher peak viral loads than those without CMV disease, corresponding to a 1.58 $\log_{10}$ difference ($P < .0003$) between the median peak loads. This finding is similar to the difference between median peak loads observed in our previous studies of transplant recipients [Cope et al., 1997a, 1997b].

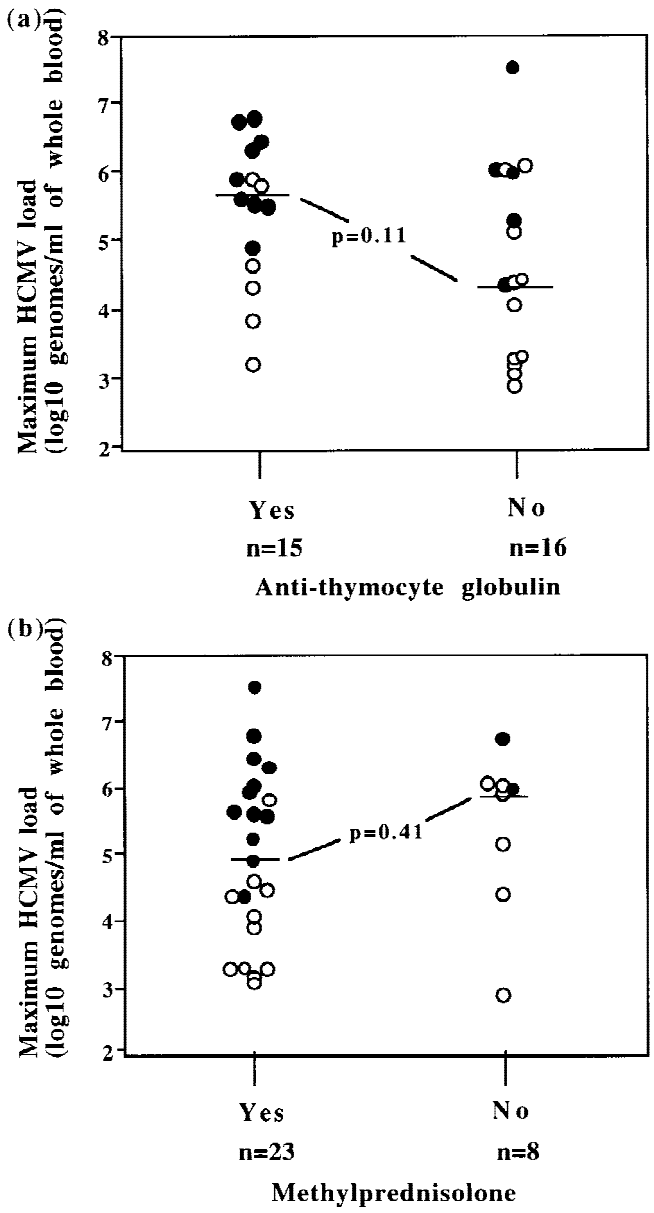

Fig. 3. Scatter diagrams showing the relationship between the maximum cytomegalovirus (CMV) load in blood from renal allograft recipients and **(a)** administration of antithymocyte globulin (ATG) and **(b)** methylprednisolone. (●) denotes patients with CMV disease.

A significant difference was observed between the median peak CMV loads from patients in the D+R+ and D+R– serostatus groups with that from the D–R+ group ($P < .05$ and $P < .01$, respectively, Mann–Whitney test). D+R+ patients had an intermediate risk of disease compared with individuals experiencing a primary CMV infection (D+R–) and patients with CMV reactivation (D– R+) [Grundy et al., 1988; Cope et al., 1997a, 1997b]. The median peak load in the D+R+ group was not significantly different from the D+R–

TABLE I. Univariate and Multivariate Analyses of Risk Factors for CMV Disease in Renal Allograft Recipients

Analysis	Odds ratio (OR)	95% Confidence interval	*P*
Univariate			
Donor positive	5.5	0.91–33.18	0.06
Recipient positive	0.17	0.03–0.89	0.04
CMV load (per 0.25 $\log_{10}$ increase)	1.66	1.16–2.36	0.005
Receipt of methylprednisolone	2.75	0.46–16.59	0.27
Receipt of ATG	4.50	0.97–20.83	0.05
Multivariate			
Donor positive	0.4	0.01–11.62	0.6
CMV load (per 0.25 $\log_{10}$ increase)	1.78	1.06–3.00	0.03
Recipient positive	1.11	0.13–9.73	0.93
CMV load (per 0.25 $\log_{10}$ increase)	1.67	1.14–2.44	0.008
Receipt of ATG	3.55	0.43–29.55	0.24
CMV load (per 0.25 $\log_{10}$ increase)	1.65	1.14–2.39	0.008

CMV, cytomegalovirus; ATG, antithymocyte globulin.

TABLE II. Sensitivity and Specificity of the QC-PCR Assay at Various Viral Load Strata

Cut-off values	Number of patients below cut-off	Number of patients above cut-off	Sensitivity of QC-PCR assay	Specificity of QC-PCR assay
3.5	6	25	100%	33%
4.0	7	24	100%	39%
4.5	12	19	100%	67%
5.0	14	17	92%	72%
5.5	16	15	85%	78%
6.0	23	8	46%	89%
6.6	28	3	23%	100%

QC-PCR, quantitative competitive-polymerase chain reaction.

group ($P > .05$). The data shown here are consistent with previous findings [Cope et al., 1997a, 1997b; Gor et al., 1998] in that patients experiencing a primary CMV infection have CMV disease due to elevated CMV levels, once again indicating the importance of CMV load in the pathogenesis of CMV.

Univariate analysis suggested that donor seropositivity (OR 5.5) was associated with CMV disease and recipient seropositivity (OR 0.17) had a protective effect. With respect to immunosuppressive therapy, only ATG was significantly associated with an increased risk of CMV disease in univariate analyses (OR 4.5, $P = .05$), although methylprednisolone was also associated with an elevated risk (OR 2.75, $P = .27$). In multivariate logistic regression analyses, donor seropositivity, recipient seropositivity, and receipt of ATG all became nonsignificant after adjusting for CMV load, indicating that the major risk factor for CMV disease appears to be increases in CMV load and that these risk factors are mediated via an increase in viral load. In contrast, it has been shown in liver transplant recipients that the administration of methylprednisolone and high CMV load are independent risk factors for CMV disease [Cope et al., 1997b]. The difference may reflect the increased usage of methylprednisolone to control rejection in liver transplant recipients, or that the present study lacks statistical power. Indeed, there may be a two-way effect of these agents in this population with ATG acting to increase viral replication, whilst methylprednisolone acts to increase the host's susceptibility to CMV disease at a given viral load. To disentangle these complex interactions will require analysis of more patients, but it is interesting to note that patients who received ATG had higher peak viral loads (i.e., ATG increased viral replication), whereas patients who received methylprednisolone had lower peak viral loads (i.e., methylprednisolone increased the host's susceptibility to disease) in comparison with the respective untreated patients.

One approach in treating CMV disease post-transplant is via pre-emptive therapy with antivirals such as ganciclovir. Studies such as this stress the importance of not only routinely monitoring renal transplant patients for CMV viraemia, but also quantifying CMV DNAemia. This monitoring is of particular importance in those individuals who are at most risk of disease. If viral loads go above threshold levels, then antiviral therapy could be initiated to prevent disease by lowering viral replication, and hence reducing viral levels below these threshold values. We suggest that it is important to monitor the CMV DNA load of patients when they are shown to have active CMV infection by qualitative PCR. Although patients with high viral loads are frequently symptomatic, it is possible that by assessing longitudinal viral load profiles, earlier viral loads may also be a useful prognostic marker. These

approaches are being evaluated currently at our centre.

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