The Dynamics of Human Cytomegalovirus Replication In Vivo

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Summary

Cytomegalovirus (CMV) is generally described as a slowly replicating virus. During studies of immunocompromised patients, we observed rapid changes in the quantity of CMV DNA present in serial blood samples by quantitative-competitive polymerase chain reaction commensurate with a doubling time of <2 d. To further investigate the dynamics of replication in vivo, patients in three distinct situations were studied in detail: (a) those receiving intravenous ganciclovir; (b) those in whom ganciclovir-resistant strains appeared during long-term therapy; and (c) those in whom ganciclovir-resistant strains disappeared with alternative drug therapy. In all cases, it was possible to provide accurate estimates of the doubling time of CMV and its half-life of disappearance after antiviral chemotherapy. The results from all three approaches demonstrated that the doubling time/half-life of CMV in blood is ~1 d when frequent samples are collected. These results show that CMV DNA replication in vivo is a highly dynamic process. We conclude that the reputation of CMV as a slowly replicating virus based on the time taken to produce cytopathic effects in vitro is unwarranted. These findings have implications for the potency, dose, and duration of antiviral chemotherapy needed for the effective treatment of this important human pathogen.

Key words: polymerase chain reaction • quantitation • ganciclovir • mutation • fitness

Human CMV, a member of the *Betaherpesvirinae*, infects ~60% of individuals in the developed world and >90% of individuals in the developing world (1). In the immunocompetent individual, infection is usually asymptomatic, but in hosts whose immunity is impaired, such as the neonate, the transplant recipient, or HIV-infected individuals, the full pathogenic potential of the virus may be realized (1). Diseases associated with CMV infection include pneumonitis, hepatitis, gastrointestinal tract disease, and retinitis.

CMV has been generally regarded as a slowly replicating virus on the basis of time to appearance of cytopathic effect in cell culture, especially when compared with other members of the *Herpesviridae* such as herpes simplex virus type 1 (2). Studies to date in humans suggest that, after initial infection, CMV remains in a latent state in monocytes, and recent data have shown that allogeneic stimulation of monocytes from CMV-seropositive individuals can lead to viral replication and viral antigen expression (3). In the immunocompromised host, infection with CMV strains from the donor organ (4, 5) or reactivation of recipient strains (6) can occur leading to increases in viral load in the blood and commensurate disease development. Our group, among others, has shown in longitudinal analyses of transplant recipients and AIDS patients that CMV load is an important parameter in pathogenesis, such that symptomatic patients have a consistently higher median viral load than asymptomatic individuals (7–10). Furthermore, multivariate statistical models demonstrate that previously recognized risk factors for disease, such as CMV serostatus of the donor and recipient, are explained through elevated viral load in the posttransplant period (7–9).

During these investigations, we observed that CMV loads increased markedly during active infection of immunocompromised hosts and wished to ascertain the dynamics of viral replication in vivo. Using an approach that recently defined the highly dynamic nature of HIV and particularly hepatitis C virus (HCV)[1] replication (11–13), we exploited the ability of potent antiviral intervention to perturb the host–virus equilibrium, together with population dynamics (14), to provide for the first time an estimate of the doubling time of CMV in the human host. Since estimates derived from one strategy could be misleading, we used two further approaches to obtain estimates of the dynamics of CMV replication in vivo; all of these results concur and indicate

[1]*Abbreviations used in this paper:* GCV, ganciclovir; HCV, hepatitis C virus; HPMPC, cidofovir; PMA, point mutation assay.

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that CMV replicates dynamically in the immunocompromised host.

Materials and Methods

Patients Investigated

The results calculated have come from detailed investigations of patient cohorts whose clinical features have been described elsewhere (8, 9, 15, 16).

Extraction of CMV DNA and PCR Conditions

DNA was extracted from whole blood samples (200 μl) using ion exchange chromatography with a Qiagen DNA extraction kit according to the manufacturer's instructions. Primers directed towards the CMV glycoprotein B (UL55) gene were used for the qualitative and quantitative detection of CMV and have been described in detail elsewhere (17). The quantitative-competitive PCR method for assessing CMV load used an internal control sequence consisting of a mutagenized version of the 149-bp authentic target sequence. The details of this method have been described extensively elsewhere (7–9, 18). The lowest level of detection in these assays is 200 genomes/ml blood.

Detection of UL97 Mutants in Clinical Samples

The following primers were used for the amplification of UL97: primer 1, (outer) 5′ AGACGGTGCTACGGTCTGGATGT; primer 2, (outer) 5′ GTTTGTACCTTCTCTGTTGCCTTT; and the nested primers: primer 3, (inner) 5′ CAACGTCACGGTACATCGACGTTT; primer 4, (inner) 5′ GCCATGCTCGCCCAGGAGACAGG.

The nested amplification was performed using conditions described previously (19) and yielded a 700-bp amplicon.

The microtiter point mutation assay (PMA) for the detection of UL97 mutations at codons 460, 520, 594, and 595 has been described in detail elsewhere (19).

Analysis of Results

Response of CMV Load to Ganciclovir Therapy. The basic models used for viral dynamics of CMV are similar to those described for HIV (11–13, 20–22). Assuming that virus DNA levels in blood after therapy with ganciclovir (GCV) decline according to an exponential function, the slope of decline can be used to calculate the rate of viral clearance in blood. If the system is in equilibrium at initiation of therapy with the rate of viral production equal to the rate of viral clearance, then after therapy with a drug that totally blocks viral production, the dynamics of GCV is given by $y(0)e^{-at}$, where $y(0)$ is the initial viral load and a is the viral clearance rate constant. Plotting the change in CMV viral load with time followed by computation of the slope of decline after initiation of GCV therapy allows the half-life of virus in the blood to be calculated according to the formula $t_{1/2} = -\ln 2/\text{slope}$.

Fitness of UL97 Variants and Generation Time. The relative fitness, s, of the population (in this case, different UL97 variants) was calculated from the following equation, which assumes replication occurs in continuous time (23). This equation merely requires knowledge of the relative proportion of the most fit variant (p) and the least fit variant (q) at times 0 and t, respectively. These quantitative parameters were obtained from the PMA described above.

$$s = \frac{1}{t}\ln\frac{\lceil q(t)p(0)\rceil}{\lfloor p(t)q(0)\rfloor} \quad (1)$$

In some cases, the proportions of the genotypes in the $p(0)$ or $q(0)$ category were below the detection limit of the PMA, and so this value was estimated to calculate the relative fitness gain of mutant virus in the presence of GCV. In such cases, different estimates for the proportion of mutant genotypes were used but generally did not substantially affect the calculated fitness gain. Simulated repopulation curves of wild-type or mutant UL97 genotypes were generated by computing the proportion of $p(t)$ at daily intervals with different starting populations of q.

Results

Decay Rates of CMV DNA after Antiviral Intervention

The first approach to assess the dynamics of viral replication was to perturb the host–viral equilibrium by introducing a potent inhibitor of replication and then to determine the rate of decline of virus in the host. Initially, we used data from 35 AIDS patients with first episode CMV retinitis who received intravenous GCV therapy for 21 d. The median CMV load in these patients at baseline was 4.95 $\log_{10}$ genomes/ml and by day 21 the majority of patients had levels of CMV DNA below the sensitivity of the assay (<200 genomes/ml; see Fig. 1) corresponding to a mean decrease of −2.46 logs. Thus, calculation of the half-life of clearance in these patients provided a conservative (upper) estimate of the half-life of decline of CMV in blood of 2.56 ± 0.36 d.

To obtain further estimates for the decline of CMV DNA after GCV intervention, we performed similar analyses in bone marrow transplant recipients (n = 11) and liver transplant recipients (n = 13) with active CMV infection. The half-life of decline of viral DNA in the blood of these patient groups after intravenous GCV therapy was 1.52 ± 0.67 d for the bone marrow recipients and 2.36 ± 1.2 d for the liver transplant recipients (data not shown).

Further refinement of the half-life of decline of CMV DNA in blood was achieved by enrolling five AIDS patients with CMV retinitis into an intravenous GCV induction study that involved frequent sampling (median of five

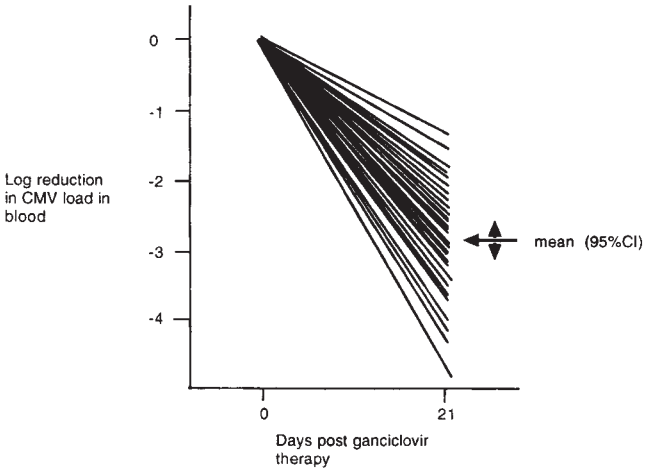

Figure 1. Log reduction in CMV load in the blood of 35 AIDS retinitis patients during 21 d of induction therapy with intravenous GCV (5 mg/kg/bd). CI, confidence interval.

samples per patient over 21 d of therapy). The CMV load modulations in these patients are shown in Fig. 2, together with the slope of decline of viral load. The average half-life of decline of CMV in the blood of these patients was 0.98 ± 0.3 d. A similar estimate was obtained for the decline of CMV DNA in the urine (0.96 ± 0.14 d; data not shown). The advantage of performing these detailed studies in the HIV-infected host relates to their relatively stable CMV DNA load (mean difference between samples before therapy 0.2 $\log_{10}$ genomes/ml) in the month preceding therapy, i.e., they fulfill the requirement for a steady state to be present at the initiation of therapy.

Kinetics of CMV Synthesis In Vivo

To provide a direct estimate of the doubling time of CMV in vivo, we studied a group of bone marrow transplant patients in whom frequent surveillance samples (median sampling time once per week) were collected after transplant and in whom CMV DNA appeared and increased in level. CMV load in the blood of 18 patients was analyzed (Fig. 3), and the mean doubling time of CMV in the blood was calculated as 2.09 ± 1.33 d (median 1.5 d, range 0.38–4.7).

Population Dynamics of Wild-type and Mutant Forms of CMV in the UL97 Gene after Antiviral Therapy

We next examined the genotypic composition of the CMV UL97 gene in AIDS patients receiving long-term antiviral therapy. Calculation of the relative fitness of mutant and wild-type virus requires a method to quantitate the relative distribution of wild-type and mutant virus present within an evolving population (see Materials and Methods). We have developed a PMA for the most frequently observed mutations in UL97 and used the data to determine the relative fitness of drug-resistant and wild-type virus.

Appearance of Drug-resistant UL97 Mutants. 10 AIDS patients treated with GCV were investigated longitudinally for the appearance of alterations in the genotypic composition of UL97 by the PMA. We calculated the relative fitness gain of the mutant UL97 population over the wild-type population using a standard formula for the effects of selection at a single locus in an asexual haploid population, as previously used for calculations of relative fitness of HIV drug-resistant mutations. Assuming replication in continuous time, the selection coefficient *s* is given by equation 1 in Materials and Methods.

Reappearance of Wild-type UL97 Genotypes. In addition to the 10 patients in whom the fitness gain of mutant virus was calculated, we also obtained samples from patients who had been exposed to GCV, had developed high-level genotypic resistance in UL97, and who were then given cidofovir (HPMPC) therapy. Since HPMPC is a phosphonate which does not require UL97 for activation, there would be no growth advantage for CMV strains with GCV-resistant UL97 genotypes. Consequently, in a mixed population, the wild-type UL97 genotype should repopulate at the expense of the mutant genotype if the virus carrying the mutant genotype is less fit. We analyzed three patients prescribed GCV followed by HPMPC and calculated the relative fitness gain of wild-type UL97 virus compared with mutant UL97 virus. A representative picture of the repopulation

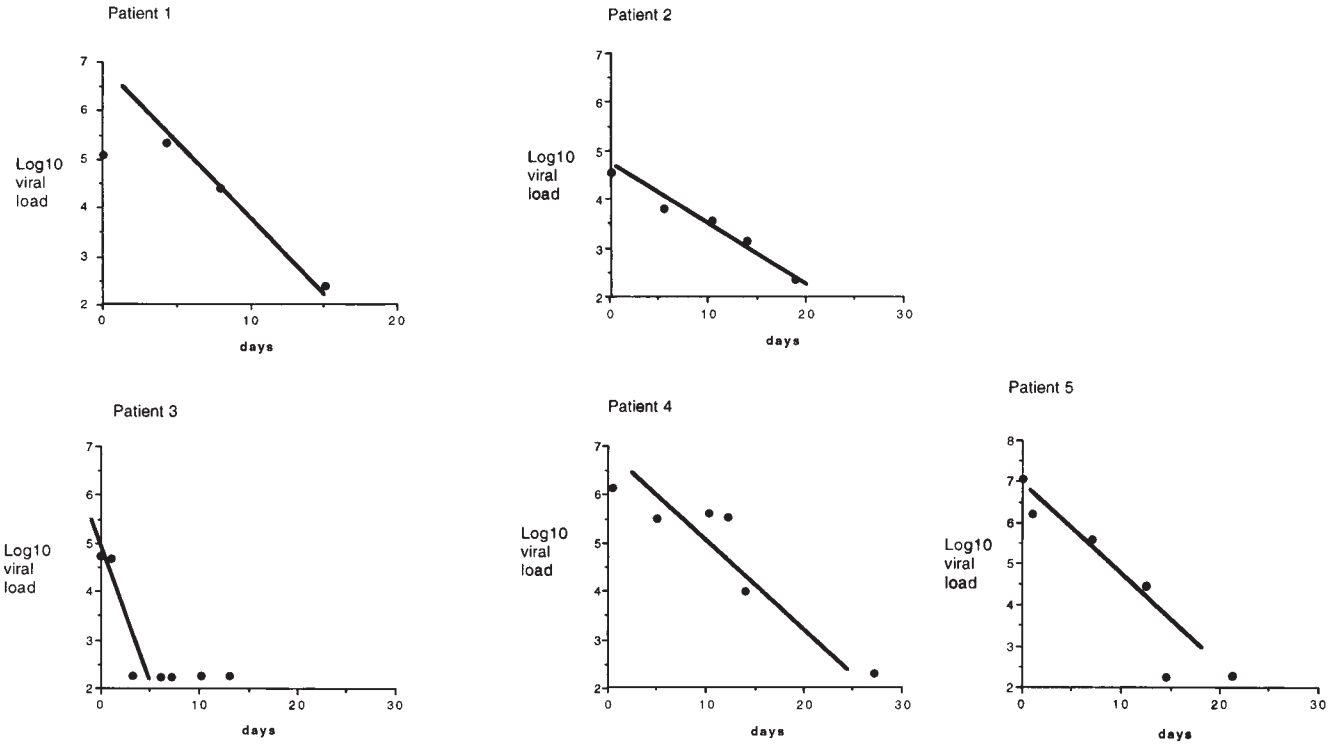

Figure 2. Reduction in CMV load in the blood of five AIDS retinitis patients subjected to intensive sampling after initiation of intravenous GCV therapy. The data points are represented as filled circles, and the computed line of best fit is shown.

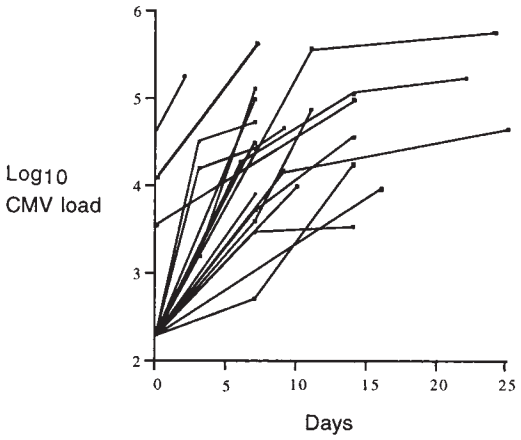

Figure 3. Rate of increase in CMV load in the blood of 18 bone marrow transplant recipients after allogeneic transplantation.

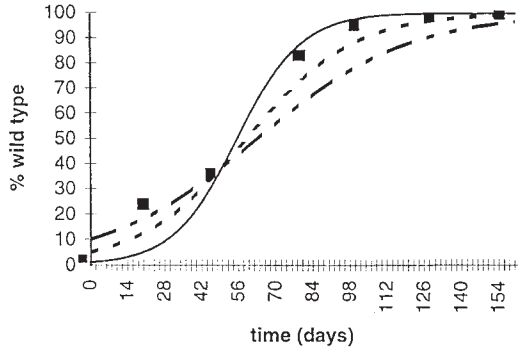

Figure 4. Simulated repopulation rates of wild-type UL97 CMV strains for the mutation L595S. The proportion of wild-type is shown for three different starting populations of wild-type genotype (1% [solid line], 5% [dashed line], and 10% [broken line] corresponding to $q(0)$ of 0.01, 0.05, and 0.1, respectively) at the initiation of HPMPC therapy. The results from the PMA are shown as filled squares.

rates of wild-type virus after HPMPC therapy is shown in Fig. 4. Similar repopulation plots were generated for all the patients under consideration.

The results generated by the analysis summarized above are shown in Table I. The fitness gain of CMV strains carrying UL97 mutations associated with GCV resistance in the presence of GCV ranged from 2.6% for the double mutant A594V + M460I to 9% for L595S. Indeed, the consistency of estimates was demonstrated for the L595F mutation, where the three patients investigated produced values of 3.9, 5.3, and 5.9% relative fitness gain. In patients whose therapy was changed from GCV to HPMPC, the relative fitness gain of wild-type viral genotypes was comparable, ranging from 3.5% for the L595S mutation to 12.8% for the double mutant M460I + L595F.

Discussion

To date, there have been no estimates of the replication rate of CMV in the human host. To gain insight into CMV replication in vivo, we used three approaches which have been extensively applied to ascertain the dynamics of HIV and HCV (11–14, 20–22). First, we assessed the rate of decline of CMV in the blood after GCV therapy in AIDS patients, liver transplant, and bone marrow transplant recipients. GCV is a potent inhibitor of the viral DNA polymerase and thus inhibits formation of new virus particles (24). The computed upper estimate of the half-life of virus in the blood was between 1.5 and 2 d. In a dynamic situation, the accuracy of these half-life estimates partially reflects the frequency of sampling; therefore, we performed frequent viral load measures in the blood of five AIDS patients, and the results refined the estimated half-life of the virus in the blood to ~1 d. Since GCV is unlikely to be 100% effective at inhibiting replication, this estimate is likely to be a minimal one and parallels the data recently presented for HCV responses after IFN therapy (13). The second approach used the direct assessment of viral load kinetics in bone marrow transplant patients undergoing active infection. The results, albeit limited by the frequency of sampling, indicated that the doubling time of CMV in the blood was ~2 d. The third approach exploited the kinetics of appearance of different genotypes as a consequence of the growth advantage provided to drug-resistant variants in the presence of selective drug pressure or to wild-type variants when the selective pressure was removed. Taken together, these approaches have shown that CMV replication in vivo is a highly dynamic process and likely proceeds with a doubling time of ~1 d.

The highly dynamic nature of certain human viral infections such as HIV, HCV, and HBV has been established using similar approaches and assumptions to those used in this study (11–14, 20–22, 25). The half-life of CMV in the blood

Table I. *Relative Fitness of Mutant and Wild-type UL97 Viruses after Initiation of Ganciclovir Therapy (Patients 1–10) or HPMPC Therapy (Patients 11–13)*

Patient	Mutation	Relative fitness gain
Mutant vs. wild-type		(%)
1	L595F	3.9
2	L595F	5.6
3	L595F	5.3
4	L595S	9.0
5	L595S	8.0
6	M460V	3.7
7	M460I	4.8
8	H520Q	5.0
9	A594V + M460I	2.6
10	L595F + M460I	7.5
Wild-type vs. mutant		
11	L595S	3.5
12	L595F	5.6
13	M460I + L595F	12.8

is more similar to the half-life of the HIV-infected lymphocyte (mean $t_{1/2}$ = 2 d) than the HBV- or HCV-infected hepatocyte ($t_{1/2}$ = 10–100 d). Immune clearance mechanisms, including lysis of infected cells by cytotoxic T lymphocytes, have been implicated in the clearance of HIV-1–infected lymphocytes (22) and can be invoked to account for the clearance of CMV-infected cells after GCV therapy (26). However, other nonspecific immune clearance mechanisms may also be instrumental in removing the CMV-infected cells. Comparison of the dynamics of CMV replication in different patient groups should provide insight into the immunologic control mechanisms operational in each host and help elucidate the relative contributions of population dynamics and immune control to preventing CMV disease.

The quantitative distribution of wild-type and mutant alleles determined by the PMA was crucial to assess the fitness of UL97 variants under GCV selection and the repopulation rates of wild-type strains after change of therapy to HPMPC, which does not require UL97 for activation (27). Importantly, none of the strains investigated had drug-resistant mutations in the UL54 gene (data not shown), and so we are confident that the fitness differences observed reside within the UL97 gene. In AIDS patients, CMV retinitis usually occurs after an extensive period of active replication (28, 29) in which many variants may be produced. The dynamics of resistance have been succinctly described for HIV by Bonhoeffer et al. (22) and are directly relevant to the dynamics of CMV resistance. The expected pretherapy frequency of mutants depends upon the number of point mutations between wild-type and mutant virus, the mutation rate associated with virus replication, the relative replication rates of wild-type virus and resistant virus, and the population size. In the case of CMV UL97 mutants, the majority of resistance mutations involve single point mutations (30, 31) and are frequently observed in AIDS patients on long-term GCV therapy in whom substantial replication will have occurred before antiviral intervention. Although the mutation rate of the CMV DNA polymerase is much lower than HIV reverse transcriptase, the population size at initiation of therapy is large and so, perhaps not surprisingly, we found evidence for small but reproducible amounts of mutant virus present at baseline, i.e., before GCV therapy. Therefore, we surmise that mutants at the UL97 loci are present within the population at a low frequency before therapy rather than generated during therapy. This may help to explain why a high frequency (70%) of AIDS patients who experience CMV viremia during therapy with GCV have evidence of UL97 mutations (16).

The relatively rapid replication rate of CMV in vivo appears to contrast with the dogma from in vitro experiments that CMV is a slowly replicating virus (2). This conclusion was reached many years ago and was based on the time to appearance of cytopathic effect and release of virions, and our data question the relevance of these observations to the in vivo situation.

In conclusion, the long-standing reputation of CMV as a slowly replicating virus requires reappraisal in light of modern molecular approaches to quantitate DNA replication. Certainly, the time to appearance of cytopathic effect in vitro is long, but this must be due to factors other than a slow DNA replicative cycle. The finding that CMV replication in vivo is a highly dynamic process has profound implications for the potency, dose, and duration of antiviral therapy required to control CMV; we suggest that the concepts (32) recently gleaned from the study of HIV should be applied to CMV.

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