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Lack of Emergence of Cytomegalovirus UL97 Mutations Conferring Ganciclovir (GCV) Resistance following Preemptive GCV Therapy in Allogeneic Stem Cell Transplant Recipients

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Fifty allogeneic stem cell transplant recipients were enrolled in a prospective cytomegalovirus pp65 antigenemia-guided preemptive therapy trial. Among these, 10 of 34 patients who received ganciclovir exhibited sustained and/or recurrent antigenemia despite treatment. Thirteen leukocyte preparations from these 10 subjects were screened for the presence of the most frequent cytomegalovirus UL97 mutations conferring ganciclovir resistance. None of these mutations were detected after mean and median ganciclovir exposures of 31.6 and 28.0 days, respectively.

Cytomegalovirus (CMV) infections represent a serious threat in patients undergoing allogeneic bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) (15). In order to reduce the morbidity and mortality associated with CMV disease, most centers have now adopted one of the two following preventive strategies. The first approach, referred to as universal prophylaxis, is based on the administration of a potent anti-CMV drug, usually ganciclovir (GCV), to all patients for the first 3 months after transplantation. Prophylaxis with GCV has been reported to be highly effective in reducing the occurrence of early CMV disease (prior to day +100) but is also associated with an increased incidence of bacterial and fungal infections and with significant bone marrow toxicity (4, 13). Long-term use of GCV has raised concerns about the development of delayed CMV disease and the possibility of selecting GCV-resistant strains (11, 25). The second strategy, called preemptive or early treatment, is based on early identification of patients with active CMV blood infections using sensitive detection assays followed by antiviral treatment for various lengths of time.

A successful CMV pp65 antigenemia-guided preemptive strategy for allogeneic hematopoietic stem cell recipients was recently reported (5). In that study, 50 patients with hematologic malignancies received a transplant (not T cell depleted) from matched sibling donors. The subjects were monitored weekly for the presence of CMV antigens and DNA (COBAS AMPLICOR CMV MONITOR test; Roche Diagnostics, Laval, Canada) in polymorphonuclear leukocytes (PMNL) from initiation of the conditioning regimen until day +98 after transplantation. Among these 50 patients, 34 (68%) were treated with GCV (14 days of intravenous [i.v.] administration [5 mg/kg of body weight twice a day] followed by 14 days of oral administration [1,000 mg three times a day]) upon a positive pp65 antigenemia assay. Eight (23.5%) of these 34 patients received a second course of therapy based on the reemergence of CMV pp65 antigens in the blood. Only one patient (2%) enrolled in the study developed CMV disease despite a negative antigenemia test. To further support the use of such a preventive approach, we now report the results of genotypic markers of CMV resistance to GCV among the same cohort of patients.

Among samples from the 50 patients enrolled in our study, 13 from 10 patients were selected for genotypic analysis on the basis of either a positive PCR for CMV in PMNL after the patient had received at least 14 days of i.v. GCV during the first treatment course (5 samples) or a second positive test for CMV DNA in the blood within the first 98 days following transplantation (8 samples). In both cases, the last PCR-positive samples were examined for genotypic evidence of resistance. The subjects who had no positive PCR throughout the study or whose CMV DNA rapidly cleared within 14 days of the first GCV treatment were assumed not to be infected with a GCV-resistant virus. The samples were collected either after or during GCV therapy (mean, 31.6 days; median, 28.0 days; range, 14 to 56 days of cumulative GCV therapy). The mean viral DNA load as determined by the COBAS AMPLICOR CMV MONITOR test for those samples was 9.96×10^3 CMV DNA copies per 4×10^6 PMNL. The presence of various CMV UL97 mutations at codons 460, 520, 594, and 595 was assessed as previously described (12, 14). Briefly, two regions (nucleotides 1088 to 1587 and 1713 to 1830) of the CMV UL97 gene were amplified using a nested-PCR protocol. Amplicons were then digested separately with enzymes *Nla*III and *Alu*I (500-bp amplicon) as well as with *Hha*I, *Taq*I, and *Mse*I (118-bp amplicon). Restriction patterns were then visualized on an 8% polyacrylamide gel stained with ethidium bromide.

Two samples from the same patient (collected after 28 and 35 days of cumulative GCV therapy) had a restriction pattern almost compatible with a Q520 mutation (Table 1). Upon

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TABLE 1. CMV load and UL97 mutations in leukocytes of allogeneic blood transplant recipients with persistent or recurrent antigenemia despite GCV preemptive therapy

Patient	No. of days after transplantation (viremia episode)	Cumulative no. of days of GCV treatment (route)	Antiviral regimen at time of sampling (route)	Viral load in PMNL by:		UL97 mutation detected[c]
				pp65 antigenemia assay[a]	PCR[b]	
1	98 (first)	14 (i.v.), 35 (p.o.[d])	GCV (p.o.)	0	1.63×10^3	None
2	63 (first)	14 (i.v.)	GCV (i.v.)	0	1.13×10^3	None
3	91 (second)	14 (i.v.), 14 (p.o.)	None	8	1.17×10^4	None
4	77 (second)	14 (i.v.), 14 (p.o.)	None	1	2.51×10^2	None
5	84 (first)	14 (i.v.), 12 (p.o.)	GCV (p.o.)	0	2.90×10^3	None
6	84 (second)	14 (i.v.), 14 (p.o.)	None	0	8.56×10^2	None
7	77 (first)	14 (i.v.), 7 (p.o.)	GCV (p.o.)	0	8.31×10^2	None
8	77 (second)	28 (i.v.), 14 (p.o.)	None	18	1.65×10^4	None
	91 (second)	42 (i.v.), 14 (p.o.)	GCV (i.v.)	0	8.80×10^2	None
9	70 (second)	14 (i.v.), 14 (p.o.)	None	1	1.88×10^4	S510
	77 (second)	21 (i.v.), 14 (p.o.)	GCV (i.v.)	2	5.64×10^4	S510
10	70 (first)	14 (i.v.), 14 (p.o.)	GCV (p.o.)	0	4.59×10^2	None
	84 (second)	14 (i.v.), 14 (p.o.)	None	4	1.72×10^4	None

[a] CMV pp65 antigenemia assay results are expressed as the numbers of positive cells per 2×10^5 PMNL.
[b] COBAS AMPLICOR CMV MONITOR test (PCR) results are expressed as the numbers of CMV copies per 4×10^6 PMNL.
[c] A rapid screening assay detected UL97 mutations Q520, S595, and F595 as well as other mutations at codons 460 and 594. The S510 mutation has a restriction pattern similar to that of a Q520 mutation.
[d] p.o., per os.

sequencing, this specific restriction pattern was found to be caused by the presence of an Asn510Ser mutation, with a wild-type sequence at codon 520. All the other samples contained no CMV UL97 mutations at codons 460, 520, 594, and 595 by the restriction enzyme assay.

Over the past few years, some concerns have been raised regarding the selection of drug-resistant CMV strains in profoundly immunosuppressed transplant recipients. Indeed, the presence of such strains has been documented for solid organ transplant recipients (1–3, 17, 19–21, 26, 29) and BMT or PBSCT recipients (8, 9, 11, 16, 23–25, 27), although the exact frequency at which GCV resistance develops in those patients is not yet known. In theory, the use of a preemptive strategy which greatly reduces drug exposure (compared to the use of universal prophylaxis) should both decrease the incidence of drug resistance and limit antiviral-related toxicities in those patients. In addition, such an approach should also contribute to reduction of the rate of delayed CMV disease as a result of early reconstitution of a CMV-specific cellular immune response (18).

In our study, viral resistance to GCV was assessed by examining the presence of the most common UL97 mutations associated with GCV resistance directly in leukocytes of PBSCT recipients who had persistent CMV antigens or DNA in their blood after a minimum of 2 weeks of i.v. GCV or during a second viremic episode. Previous results from our group indicate that such a genotype-based screening assay is more sensitive than phenotypic (susceptibility) analyses in evaluating GCV resistance in human immunodeficiency virus-infected individuals (12). This technique also offers the advantage of detecting minor UL97 mutant subpopulations (at least 10%) mixed with wild-type strains (7) with an absolute sensitivity of 25 copies, as determined by testing for a plasmid containing the UL97 gene. The only UL97 mutation (S510) detected in our study has been previously reported to occur in susceptible CMV isolates (11) and is therefore not considered to be associated with GCV resistance. Thus, no UL97 mutations known to confer GCV resistance were identified in the 50 patients enrolled in our study (95% confidence interval, 0 to 6%). Although only 13 samples from 10 patients were tested, it could reasonably be assumed that the other patients did not harbor GCV-resistant strains in their blood since they consistently had negative PCR results or rapidly became PCR negative (within 2 weeks of therapy) and did not exhibit recurrent CMV DNA in their blood. These results suggest that the rate of emergence of viral resistance in the context of short courses of preemptive GCV therapy in allogeneic PBSCT recipients is very low. In the study by Erice et al. (11), all UL97 mutations conferring GCV resistance in blood isolates of BMT patients emerged after more than 2 months of cumulative GCV therapy preceded by acyclovir prophylaxis. In contrast, the length of drug exposure was significantly shorter in our patients (mean GCV exposure, 31.6 days; range, 14 to 56 days).

It is still possible that some other UL97 or UL54 (DNA polymerase) mutations conferring resistance to GCV were not detected by our screening assay. For example, UL97 mutations at codons 591 and 603 have been previously reported after BMT (9, 23). However, given the facts that UL97 mutations at codons 460, 520, 594, and 595 represent approximately 70% of UL97 mutations associated with GCV resistance (10) and that UL54 resistance mutations usually emerge after UL97 mutations (28), the probability of having missed a significant number of GCV resistance markers remains very low. These results are in agreement with the lack of selection of GCV-resistant CMV strains as assessed by phenotypic analyses of solid organ transplant recipients with CMV viremia who received brief courses of i.v. GCV (6) and of PBSCT recipients with rising CMV antigenemia while on preemptive GCV therapy (22). In addition, none of our patients developed clinical features suggestive of GCV resistance, such as the need for foscarnet therapy or the development of late CMV disease (19).

In conclusion, the absence of the most frequent genotypic markers for GCV resistance in this study, combined with the low incidence of CMV disease as previously reported (5),

strengthens the safety and reliability of our preemptive GCV approach for PBSCT recipients.

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