

Monitoring of Trough Plasma Ganciclovir Levels and Peripheral Blood Cytomegalovirus (CMV)-Specific CD8⁺ T Cells To Predict CMV DNAemia Clearance in Preemptively Treated Allogeneic Stem Cell Transplant Recipients

Estela Giménez,^a Carlos Solano,^{b,c} José Ramón Azanza,^d Paula Amat,^b David Navarro^{a,e}

Microbiology Service^a and Hematology and Medical Oncology Service,^b Hospital Clínico Universitario, Fundación INCLIVA, Valencia, Spain; Department of Medicine, School of Medicine, University of Valencia, Valencia, Spain^c; Clinic Pharmacology Service, Clínica Universidad de Navarra, Facultad de Medicina, Universidad de Navarra, Pamplona, Spain^d; Department of Microbiology, School of Medicine, University of Valencia, Valencia, Spain^e

It is uncertain whether monitoring plasma ganciclovir (GCV) levels is useful in predicting cytomegalovirus (CMV) DNAemia clearance in preemptively treated allogeneic stem cell transplant recipients. In this observational study, including 13 episodes of CMV DNAemia treated with intravenous (i.v.) GCV or oral valganciclovir, we showed that monitoring trough plasma GCV levels does not reliably predict response to therapy. Rather, immunological monitoring (pp65 and immediate-early [IE]-1-specific gamma interferon [IFN- γ]-producing CD8⁺ T cells) appeared to perform better for this purpose.

Ganciclovir (GCV) and its ester prodrug valganciclovir (V-GCV) are the first-line drugs for preemptive treatment of active cytomegalovirus (CMV) infection in allogeneic stem cell transplant (Allo-SCT) recipients (1). These two drugs display similar efficacies in CMV DNA clearance (2), despite the fact that V-GCV leads to higher exposure than does GCV (3). Since intracellular GCV triphosphate is the active form of this compound, the plasma GCV concentration is simply a surrogate of the drug's antiviral activity (1, 2). Monitoring trough plasma GCV levels in AIDS patients receiving oral GCV as maintenance therapy for CMV retinitis was shown to be clinically useful in predicting therapy failure and disease progression (4, 5). Nevertheless, no therapeutic concentrations of GCV have been formally defined (6). In the solid organ transplant (SOT) setting, contradictory data have been reported on the clinical value of routine pharmacokinetic monitoring of GCV (6–8). This issue remains unresolved in the Allo-SCT setting. In the current study, we monitored trough plasma GCV levels during a number of episodes of CMV DNAemia in order to determine whether there is a relationship between trough plasma GCV concentrations and the efficacy in achieving plasma CMV DNA clearance. In parallel, we prospectively enumerated CMV-specific (pp65 plus immediate-early [IE]-1) gamma interferon (IFN- γ)-producing CD8⁺ T cells in blood, as previous studies demonstrated the critical influence of an adequate expansion of these T-cell populations on CMV DNAemia clearance (9, 10).

A total number of 13 episodes of CMV DNAemia developing in 13 patients were included in the current study. None of these patients had CMV end-organ disease. The clinical and demographic characteristics of these patients are shown in Table 1. This study was approved by the review board and ethics committees of the Hospital Clínico Universitario. Each patient gave written informed consent to participate in the study.

Preemptive therapy with oral V-GCV (900 mg every 12 h) or intravenous (i.v.) GCV (5 mg/kg of body weight every 12 h) was initiated when the plasma CMV DNA load reached 500 copies/ml, as determined by the CMV real-time PCR assay (Abbott Molecular, Des Plaines, IL, USA) (11), and interrupted upon two consec-

utive negative PCR results (12). Plasma GCV levels were measured by reverse-phase high-performance liquid chromatography (HPLC) coupled with spectrofluorometric detection, as previously described (13). The limit of detection of the assay is 0.25 mg/liter, and the intra- and interday coefficients of variation do not exceed 6.65%. Plasma specimens were kept at –20°C for a maximum of 6 months and were retrieved for GCV concentration measurements. It was shown previously that GCV remains stable at this temperature for at least 6 months (13, 14). Blood samples were drawn at steady state (>1 day after the initiation of therapy) just before intake of the drug (trough level). Following the current policies at our center, blood specimens from the hospitalized patients and the outpatients were scheduled to be drawn for biochemical and hematimetric analyses, virus PCR assays, and trough cyclosporine measurements within 30 to 60 min prior to V-GCV or i.v. GCV administration.

CMV pp65 and IE-1-specific IFN- γ CD8⁺ T cells were enumerated by flow cytometry for intracellular cytokine staining, as previously described (9, 10, 12). Whole blood was simultaneously stimulated with two sets of 15-mer overlapping peptides encompassing the sequences of both proteins (1 μ g/ml/peptide). Increases in CMV-specific CD8⁺ T-cell counts from baseline levels (at the time of detection of CMV DNAemia) that reached \geq 1 cell/ μ l were considered significant (9, 10).

Nine out of the 13 episodes of CMV DNAemia included in the current study were first (initial) episodes that occurred at a median of 28 days after Allo-SCT (range, 0 to 51 days). The remaining 4 episodes were recurrences that developed at a median of 321 days after Allo-SCT (range, 144 to 766 days) (Table 2, patients 3, 9,

Received 2 April 2014 Returned for modification 15 June 2014

Accepted 22 June 2014

Published ahead of print 30 June 2014

Address correspondence to David Navarro, david.navarro@uv.es.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.02953-14

TABLE 1 Demographic and clinical characteristics of study patients

Patient no.	Sex ^a	Age (yr)	Underlying disease ^b	Conditioning regimen	HLA ^c matching	Donor type	CMV serostatus ^d	Stem cell source ^e	Acute GvHD (grade) ^f
1	F	64	AML	Nonmyeloablative	Mismatched	Related	D ⁺ /R ⁺	PB	0–II
2	M	31	AML	Myeloablative	Mismatched	Unrelated	D ⁻ /R ⁺	UCB	0–II
3	F	62	AML	Nonmyeloablative	Matched	Unrelated	D ⁻ /R ⁺	PB	0–II
4	M	49	MM	Myeloablative	Mismatched	Unrelated	D ⁻ /R ⁺	UCB	0–II
5	M	39	ALL	Nonmyeloablative	Mismatched	Unrelated	D ⁻ /R ⁺	UCB	0–II
6	F	45	NHL	Myeloablative	Matched	Unrelated	D ⁺ /R ⁺	PB	0–II
7	M	63	AML	Nonmyeloablative	Matched	Related	D ⁺ /R ⁺	PB	0–II
8	M	27	AML	Myeloablative	Matched	Related	D ⁺ /R ⁻	PB	III–IV
9	M	56	AML	Nonmyeloablative	Matched	Related	D ⁺ /R ⁺	PB	0–II
10	M	38	AA	Myeloablative	Mismatched	Unrelated	D ⁻ /R ⁺	UCB	0–II
11	F	58	ALL	Nonmyeloablative	Mismatched	Unrelated	D ⁻ /R ⁺	UCB	0–II
12	F	55	AML	Nonmyeloablative	Matched	Related	D ⁺ /R ⁺	PB	III–IV
13	F	62	NHL	Nonmyeloablative	Matched	Unrelated	D ⁻ /R ⁺	PB	0–II

^a F, female; M, male.

^b AML, acute myeloid leukemia; MM, multiple myeloma; ALL, acute lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; AA, aplastic anemia.

^c HLA, human leukocyte antigen.

^d D, donor; R, recipient.

^e PB, peripheral blood; UCB, umbilical cord blood.

^f GvHD, graft versus host disease.

10, and 13). Ten episodes were preemptively treated with i.v. GCV, and 3 were treated with oral V-GCV. All the patients had a glomerular filtration rate of >60 ml/min.

Trough plasma GCV levels were measured in 52 specimens (median, 3/patient; range, 2 to 10/patient). CMV-specific CD8⁺ T-cell levels in 79 specimens were determined (median, 5/patient; range, 2 to 13/patient). These data are shown in Table 2 and are summarized as follows. (i) CMV DNAemia clearance was achieved in 9 episodes, after a median of 24 days of treatment (range, 15 to 87 days); the remaining 4 episodes were still active at the end of the follow-up (patient death). (ii) Trough plasma GCV levels of >0.6 mg/liter were consistently measured in 10 episodes (median, 2.90 mg/liter; range, 1.17 to 5.80 mg/liter). Four of these episodes (1, 2, 4, and 5) remained unresolved at the end of the follow-up. A significant expansion of CMV-specific IFN- γ -producing CD8⁺ T cells was not demonstrated in any of these episodes. A single plasma specimen/patient from patients 1, 2, and 5

was used for sequence analysis of the *UL54* and *UL97* genes. These specimens were obtained at least 4 weeks following antiviral inception, and the specimens displayed CMV DNA loads of >1,000 copies/ml, which is approximately the limit of detection of the PCR sequencing method employed in the current study (9). A mutation known to confer resistance to GCV was found only in patient 5 (M460V in the *UL97* gene). As for the 6 episodes that were eventually cleared, a significant expansion of CMV-specific IFN- γ CD8⁺ T cells was observed in 3 episodes. As for the remaining 3 episodes (patients 8, 11, and 12), we cannot rule out the possibility that functional CD8⁺ T cells targeting other viral proteins contributed to CMV DNAemia clearance. (iii) Fluctuating trough plasma GCV levels (defined by those changing from higher than to less than 0.6 mg/liter one or more times over the study period) were observed throughout 2 episodes. These episodes were eventually cleared in concomitance with a significant expansion of CMV-specific CD8⁺ T cells. (iv) Undetectable plasma

TABLE 2 Virological, immunological, and pharmacokinetic data on 13 episodes of CMV DNAemia developing in 13 allogeneic stem cell transplant patients

Patient no.	Treatment	Trough plasma ganciclovir level		Peak CMV DNA load (copies/ml)	CMV DNAemia (clearance status/day after treatment inception)	Significant expansion of CMV-specific IFN- γ CD8 ⁺ T cells ^a	Peak level of CMV-specific IFN- γ CD8 ⁺ T cells (cells/ μ l)
		Median (range) (mg/liter)	No. of measurements				
1	Ganciclovir	5.80 (0.64–5.80)	4	3,872	No/46	No	0.58
2	Ganciclovir	2.57 (1.65–4.92)	3	3,766	No/29	No	0.80
3	Ganciclovir	<0.25 (<0.25–1.59)	4	3,221	Yes/15	Yes	91.06
4	Ganciclovir	1.17 (1.04–3.48)	4	18,075	No/18	No	0.00
5	Ganciclovir	5.48 (1.15–53.40)	6	92,823	No/97	No	0.00
6	Valganciclovir	<0.25 (<0.25–6.65)	5	13,995	Yes/33	Yes	5.15
7	Ganciclovir	<0.25	10	2,129	Yes/87	Yes	14.00
8	Ganciclovir	3.97 (1.54–4.61)	3	676	Yes/27	No	0.00
9	Ganciclovir	0.62–1.34	2	2,876	Yes/23	Yes	237.92
10	Ganciclovir	2.22 (1.51–2.93)	3	2,442	Yes/32	Yes	2.22
11	Valganciclovir	1.86 (1.55–2.00)	3	1,149	Yes/27	No	0.00
12	Valganciclovir	4.47–4.92	2	1,108	Yes/15	No	0.00
13	Ganciclovir	3.22 (2.75–3.69)	3	907	Yes/16	Yes	1.98

^a Increases in CMV-specific CD8⁺ T-cell counts from baseline levels (at the time of detection of CMV DNAemia) that reached ≥ 1 cell/ μ l were considered significant.

GCV levels were seen in 1 episode, despite the administration of i.v. GCV. This episode was eventually cleared upon a significant expansion of CMV-specific IFN- γ CD8⁺ T cells. (v) In patients 3, 9, and 13, an expansion of CMV-specific CD8⁺ T-cell levels of >1 cell/ μ l was achieved at the time of plasma CMV DNAemia clearance, whereas in patients 6, 7, and 10, it preceded CMV DNAemia clearance by 1 week (patients 6 and 10) or 3 weeks (patient 7). Variability in the time course of reaching a sufficient T-cell expansion to control active CMV infection was previously reported by our group (10).

There is scant information on whether the measurement of plasma GCV levels is of any clinical utility in predicting the response to preemptive therapy in Allo-SCT recipients. In fact, the therapeutic range of plasma GCV concentrations has not been formally established in any clinical setting, although trough levels between 0.2 and 2.0 mg/liter in solid organ transplant recipients with CMV end-organ disease and >0.6 mg/liter in HIV patients with retinitis have been tentatively proposed (4–6, 15). In this context, we found that maintained trough plasma GCV levels of >0.6 mg/liter were not consistently associated with CMV DNAemia clearance. In none of the unresolved episodes was a significant expansion of CMV-specific IFN- γ -producing CD8⁺ T cells observed. These findings are in keeping with data reported by Emery et al. (16), which revealed that an anti-CMV drug with an efficacy of >93% is required to eliminate viral growth during infection of CMV-naïve liver transplant recipients, whereas lower efficacy levels (84%) are sufficient to reduce the basic reproductive number (R_0 value) to <1 in experienced CMV-immune hosts. In contrast, either undetectable or fluctuating trough plasma GCV levels were observed throughout 4 episodes that were nevertheless cleared, most likely at the expense of a robust expansion of CMV-specific IFN- γ -producing CD8⁺ T cells of >1 cell/ μ l, previously shown to be associated with viral clearance (9, 10). This T-cell expansion was manifest at the time of the first negative real-time PCR result or shortly before it. These data further prove the critical role of CMV-specific functional T-cell immunity in controlling CMV replication. In addition, maintained trough levels of >0.6 mg/liter were observed in 3 episodes in which a concomitant expansion of CMV-specific IFN- γ -producing CD8⁺ T cells was demonstrated, so we were unable to ascertain the relative contribution of each factor to viral clearance. Taken together, our data are in line with those of Perrottet et al. (7), who reported variable CMV DNAemia clearance (in whole blood) despite adequate plasma levels during V-GCV treatment for CMV disease in donor-positive/recipient-negative SOT recipients.

For the current study, we used leftover plasma specimens which were submitted to our laboratory for routine CMV DNA load monitoring (performed once a week). Thus, we were unable to determine the pharmacokinetic parameters, such as the maximum concentration of drug in serum (C_{max}), the area under the concentration-time curve from 0 to 12 h (AUC_{0-12}), and the AUC_{0-24} , whose clinical utility in predicting response to therapy has been proposed (6, 8, 15, 17). In addition, the unavailability of a number of plasma specimens precluded a more systematic analysis of GCV levels throughout the duration of the episodes. Despite these limitations, our data indicate that monitoring trough plasma GCV levels does not reliably predict the response to therapy in this clinical setting. Rather, immunological monitoring appeared to perform better for this purpose. On the basis of our data, prospective studies involving larger cohorts of patients on an ad-

equated sampling schedule for GCV measurements seem to be warranted.

ACKNOWLEDGMENTS

This research was supported by grants 09/1117 and 12/1992 from the Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo, Spain.

Paula Amat is a research fellow of the Asociación Española Contra el Cáncer (AECC).

We declare no conflicts of interest.

REFERENCES

- Solano C, Navarro D. 2010. Clinical virology of cytomegalovirus infection following hematopoietic transplantation. *Future Virol.* 5:111–124. <http://dx.doi.org/10.2217/fvl.09.64>.
- van der Heiden PL, Kalpoe JS, Barge RM, Willemze R, Kroes AC, Schippers EF. 2006. Oral valganciclovir as pre-emptive therapy has similar efficacy on cytomegalovirus DNA load reduction as intravenous ganciclovir in allogeneic stem cell transplantation recipients. *Bone Marrow Transplant.* 37:693–698. <http://dx.doi.org/10.1038/sj.bmt.1705311>.
- Einsele H, Reusser P, Bornhäuser M, Kalhs P, Ehninger G, Hebart H, Chalandon Y, Kröger N, Hertenstein B, Rohde F. 2006. Oral valganciclovir leads to higher exposure to ganciclovir than intravenous ganciclovir in patients following allogeneic stem cell transplantation. *Blood* 107:3002–3008. <http://dx.doi.org/10.1182/blood-2005-09-3786>.
- Piketty C, Bardin C, Gilquin J, Mahe V, Kazatchkine MD, Chast F. 1996. Low plasma concentrations achieved with conventional schedules of administration of ganciclovir in patients with AIDS. *J. Infect. Dis.* 174:188–190. <http://dx.doi.org/10.1093/infdis/174.1.188>.
- Piketty C, Bardin C, Gilquin J, Gairard A, Kazatchkine MD, Chast F. 2000. Monitoring plasma levels of ganciclovir in AIDS patients receiving oral ganciclovir as maintenance therapy for CMV retinitis. *Clin. Microbiol. Infect.* 6:117–120. <http://dx.doi.org/10.1046/j.1469-0691.2000.00014.x>.
- Scott JC, Partovi N, Ensom MH. 2004. Ganciclovir in solid organ transplant recipients: is there a role for clinical pharmacokinetic monitoring? *Ther. Drug Monit.* 26:68–77. <http://dx.doi.org/10.1097/00007691-200402000-00014>.
- Perrottet N, Manuel O, Lamoth F, Venetz JP, Sahli R, Decosterd LA, Buclin T, Pascual M, Meylan P. 2010. Variable viral clearance despite adequate ganciclovir plasma levels during valganciclovir treatment for cytomegalovirus disease in D⁺/R⁻ transplant recipients. *BMC Infect. Dis.* 10:2. <http://dx.doi.org/10.1186/1471-2334-10-2>.
- Bedino G, Esposito P, Bosio F, Corradetti V, Valsania T, Rocca C, Pattonieri EF, Gregorini M, Rampino T, Dal Canton A. 2013. The role of therapeutic drug monitoring in the treatment of cytomegalovirus disease in kidney transplantation. *Int. Urol. Nephrol.* 45:1809–1813. <http://dx.doi.org/10.1007/s11255-012-0293-y>.
- Tormo N, Solano C, Benet I, Clari MA, Nieto J, de la Cámara R, López J, López-Aldeguer N, Hernández-Boluda JC, Remigia MJ, García-Noblejas A, Gimeno C, Navarro D. 2010. Lack of prompt expansion of cytomegalovirus pp65 and IE-1-specific IFN γ CD8⁺ and CD4⁺ T cells is associated with rising levels of pp65 antigenemia and DNAemia during pre-emptive therapy in allogeneic hematopoietic stem cell transplant recipients. *Bone Marrow Transplant.* 45:543–549. <http://dx.doi.org/10.1038/bmt.2009.172>.
- Tormo N, Solano C, Benet I, Nieto J, de la Cámara R, García-Noblejas A, Clari MA, Chilet M, López J, Hernández-Boluda JC, Remigia MJ, Navarro D. 2010. Kinetics of cytomegalovirus (CMV) pp65 and IE-1-specific IFN γ CD8⁺ and CD4⁺ T cells during episodes of viral DNAemia in allogeneic stem cell transplant recipients: potential implications for the management of active CMV infection. *J. Med. Virol.* 82:1208–1215. <http://dx.doi.org/10.1002/jmv.21799>.
- Gimeno C, Solano C, Latorre JC, Hernández-Boluda JC, Clari MA, Remigia MJ, Furió S, Calabuig M, Tormo N, Navarro D. 2008. Quantification of DNA in plasma by an automated real-time PCR assay (cytomegalovirus PCR kit) for surveillance of active cytomegalovirus infection and guidance of preemptive therapy for allogeneic hematopoietic stem cell transplant recipients. *J. Clin. Microbiol.* 46:3311–3318. <http://dx.doi.org/10.1128/JCM.00797-08>.
- Tormo N, Solano C, Benet I, Nieto J, de la Cámara R, López J, García-Noblejas A, Muñoz-Cobo B, Costa E, Clari MA, Hernández-Boluda JC, Remigia MJ, Navarro D. 2011. Reconstitution of CMV pp65 and IE-1-specific IFN- γ CD8(+) and CD4(+) T-cell responses affording protection

- from CMV DNAemia following allogeneic hematopoietic SCT. *Bone Marrow Transplant.* 46:1437–1443. <http://dx.doi.org/10.1038/bmt.2010.330>.
13. Campanero MA, Sadaba B, García-Quetglas E, Azanza JR. 1998. Development and validation of a sensitive method for the determination of ganciclovir in human plasma samples by reversed-phase high-performance liquid chromatography. *J. Chromatogr. Biomed. Sci. Appl.* 706:311–317. [http://dx.doi.org/10.1016/S0378-4347\(97\)00666-X](http://dx.doi.org/10.1016/S0378-4347(97)00666-X).
 14. Yoshida T1, Takahashi R, Imai K, Uchida H, Arai Y, Oh-ishi, T. 2010. A simple, sensitive determination of ganciclovir in infant plasma by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. Sci.* 48:208–211. <http://dx.doi.org/10.1093/chromsci/48.3.208>.
 15. Fletcher C, Sawchuk R, Chinnock B, de Miranda P, Balfour HH, Jr. 1986. Human pharmacokinetics of the antiviral drug DHPG. *Clin. Pharmacol. Ther.* 40:281–286. <http://dx.doi.org/10.1038/clpt.1986.177>.
 16. Emery VC, Hassan-Walker AF, Burroughs AK, Griffiths PD. 2002. Human cytomegalovirus (HCMV) replication dynamics in HCMV-naive and -experienced immunocompromised hosts. *J. Infect. Dis.* 185:1723–1728. <http://dx.doi.org/10.1086/340653>.
 17. Autmizguine J, Théoret Y, Launay E, Duval M, Rousseau C, Tapiéro B, Boivin G, Ovetchkine P. 2011. Low systemic ganciclovir exposure and preemptive treatment failure of cytomegalovirus reactivation in a transplanted child. *J. Popul. Ther. Clin. Pharmacol.* 18:e257–e260.