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Resistance pattern of cytomegalovirus (CMV) after oral valganciclovir therapy in transplant recipients at high-risk for CMV infection

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ABSTRACT

In transplant recipients, cytomegalovirus (CMV) resistance to antivirals causes an increasing problem. Here we report the clinical, therapeutic, and virological characteristics of 11 cases of CMV resistance among transplant recipients at high-risk for CMV infection and receiving valganciclovir as a prophylactic, preemptive or maintenance therapy. Active CMV infection was monitored by viral DNA quantification in whole blood, and CMV resistance was assessed by UL97 and UL54 viral gene sequencing. For 10 patients, ganciclovir resistance detected after valganciclovir therapy was associated with one mutation within UL97 phosphotransferase located at codons 460 and 592–603, which constitutes a similar pattern of resistance to what has been reported previously in AIDS patients treated with valganciclovir. For the last patient, two mutations in UL97 and UL54 genes were identified. The start of valganciclovir maintenance treatment after an intravenous curative treatment while CMV DNA is still detectable in peripheral blood might represent a risk factor for the emergence of CMV resistance. The possible emergence of CMV resistance in transplant recipients at high-risk for CMV infection who receive valganciclovir therapy should be taken into account. Among those patients, CMV infection has to be closely monitored in order to detect promptly the emergence of drug-resistance.

1. Introduction

Cytomegalovirus (CMV) infections remain a major cause of morbidity after solid organ transplantation (SOT) and allogeneic stem cell transplantation (SCT). Moreover, the emergence of CMV resistance to antivirals causes an increasing problem in transplant recipients, complicating therapeutic and clinical management. In immunocompromised patients, several risk factors have been identified for the emergence of CMV resistance, such as high CMV load, prolonged antiviral therapy, or subtherapeutic doses of the antiviral (Drew et al., 2001; Emery and Griffiths, 2000). The molecular mechanisms of CMV resistance to antiviral drugs consist of the presence of mutations in the viral phosphotransferase (UL97) and DNA polymerase (UL54) genes. Four compounds are currently licensed for the prevention and/or the treatment of CMV infections in immunocompromised patients: ganciclovir, valganciclovir, foscarnet, and cidofovir. Valganciclovir, an L-valyl ester of ganciclovir with a high oral bioavailability, is the newest antiviral drug available (Reusser, 2001). In Europe, this molecule is indicated for CMV disease prevention in CMV-negative SOT recipients receiving a graft from a CMV-positive donor. Nevertheless, given the convenience to treat patients orally, without hospitalization required for intravenous treatments, valganciclovir tends to be widely used among transplant recipients, not only for prophylaxis, but also for preemptive and maintenance therapies. Several randomised prospective studies conducted in SOT recipients at risk for CMV infection have reported the safety and the efficacy of prophylactic or preemptive valganciclovir treatment, and the absence of CMV resistance mutations (Boivin et al., 2004; Humar et al., 2006; Khoury et al., 2006; Paya et al., 2004). The efficacy of oral valganciclovir, compared to intravenous ganciclovir, has been reported for preemptive therapy of CMV infections after SCT and for curative treatment of CMV disease after SOT (Asberg et al., 2007; Van der Heiden et al., 2006). However, little information is available concerning CMV resistance

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following valganciclovir therapy, since only 7 cases have so far been reported in the literature yet: 5 cases after prophylaxis in SOT recipients (Eid et al., 2008; Humar et al., 2005), and 2 cases after preemptive therapy in SCT recipients (Marfori et al., 2007). Here we describe the clinical, therapeutic, and virological characteristics of 11 different cases of CMV resistance observed in transplant recipients at high-risk for CMV infection and receiving valganciclovir therapy.

2. Patients and methods

2.1. Patients and antiviral treatments

Between 2004 and 2006, in the context of our virological diagnosis activity, we retrospectively identified 11 cases of CMV resistance occurring after valganciclovir therapy (Table 1). The group of patients (8 men, 3 women, median age = 46 years) included SOT recipients ($n = 8$) and SCT ($n = 3$) recipients. The CMV donor (D)/recipient (R) serostatus patterns were D^+/R^- for all SOT recipients and D^-/R^+ for all SCT recipients. Doses, start and stop dates of oral valganciclovir treatments are indicated in Table 1. Three different strategies were represented: prophylactic treatment during 90 days after transplantation ($n = 3$), preemptive treatment when CMV active infection was assessed by a viral load $\geq$3.0 log ($n = 3$), and maintenance treatment after intravenous curative treatment with ganciclovir +/− foscarnet ($n = 5$).

2.2. Definition of CMV infection

Symptomatic CMV infections, either CMV syndrome or CMV end-organ disease, were defined according to standard criteria previously published (Ljungman et al., 2002).

2.3. CMV infection monitoring

Active CMV infection after transplantation was monitored by viral DNA quantification in sequential whole blood samples. For the patients receiving preemptive valganciclovir therapy (#4, #5, #6), CMV infection was monitored twice a week during the first month posttransplantation, once a week during the second and third months posttransplantation, and twice a month until the sixth month posttransplantation. The median number of blood samples collected for each patient from the time of transplantation until the time of CMV resistance detection was 18 (range, 12–48) (Table 1). DNA extraction was performed with the MagNA Pure Compact Nucleic Acid Isolation kit I using the MagNA Pure Compact Instrument (Roche, Meylan, France). CMV load was then evaluated using an in-house real-time PCR assay, based on TaqMan technology, as previously described (Deback et al., 2007).

2.4. CMV genotypic antiviral resistance testing

Resistance testing was performed when the kinetics of CMV load in blood led to the suspicion of drug resistance during valganciclovir treatment (i.e., when CMV DNA was detected among patients with prophylaxis, and when no significant decrease of CMV load was observed among patients with either preemptive or maintenance therapy). CMV genotypic resistance testing was performed by UL97 and UL54 gene sequencing. DNA fragments encompassing codons 405–708 for UL97 and 269–1070 for UL54 were amplified by nested PCR. For each gene, the first PCR was performed with 10 μL of DNA extract using the proofreading enzyme Expand High Fidelity (Roche) in a mix containing 1× buffer, 1.5 mM $MgCl_2$, 800 μM dNTP and 300 μM forward and reverse outer primers (Table 2). The nested PCR was performed with 5 μL of the first PCR product using inner primers in a mix identical to that of the first PCR. For both first and nested PCR, the annealing temperatures were 55 °C for UL97, and 52.3 °C for UL54. Amplified products were sequenced with the Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Courtaboeuf, France), using the inner primers for UL97 and overlapping primer pairs for UL54 (Table 2), and analyzed with the automated sequencer ABI 3100 Genetic Analyzer (Applied Biosystems). This sequencing approach covers all reported resistance mutations in both genes. All nucleotide and amino acid sequences were compared with that of laboratory strain AD169 (GenBank accession no. BK000394) using SeqScape v2.5 software.

3. Results

The median time of onset of CMV infection after transplantation was 39 days (range: 13–118). Seven patients exhibited symptomatic CMV infections: 4 CMV syndromes and 3 CMV diseases (Table 1). Among the patients with valganciclovir prophylaxis ($n = 3$), CMV load became detectable before the end of the 90-day treatment in 2 cases. Among the patients receiving preemptive therapy ($n = 3$), valganciclovir was started when CMV load was $\geq$3.0 log. Among the recipients with maintenance therapy following intravenous curative treatment ($n = 5$), CMV load was still detectable in 3 patients when valganciclovir treatment was initiated (Table 3).

The genotypic resistance of CMV was evidenced after a median time of 145 days posttransplantation (range: 111–255). At that time, the median CMV load in whole blood was 4.7 log (range: 3.1–5.7). As far as resistance mutations are concerned, for all patients but one (#6), CMV resistance to ganciclovir was associated with the presence of one single mutation in UL97 gene, and the absence of any mutation in UL54 gene (Table 3). The majority of these mutations ($n = 7$) consisted of an amino acid substitution within the codons 592–595 of the protein. The 3 other amino acid changes observed were C603W ($n = 2$) and M460I ($n = 1$). Regarding the patient #6, 2 changes associated with CMV resistance were identified: E596G in UL97 phosphotransferase and L545S in UL54 DNA polymerase. Furthermore, amino acid modifications known as polymorphism changes were detected for all patients in DNA polymerase, but only for one patient (#6) in phosphotransferase. Lastly, 6 novel mutations of unknown phenotype were evidenced, one in UL97 phosphotransferase (V498I) and 5 in UL54 DNA polymerase (E315D, P608S, T610M, G629S, S880L).

The therapeutic management of ganciclovir-resistant CMV infections was based on the intravenous administration of foscarnet for 8 patients (#1, #3, #4, #7, #8, #9, #10, #11) and the stopping of valganciclovir treatment without starting another antiviral treatment for 3 patients (#2, #5, #6). Regarding the outcome, all patients but one are still alive: the SCT recipient #9 died with multiple organ failure on day 241 posttransplantation (Table 1).

4. Discussion

Here we report the clinical, therapeutic and virological characteristics of 11 cases of CMV resistance retrospectively identified in transplant recipients receiving oral valganciclovir as prophylactic, preemptive, or maintenance treatment. To our knowledge, this is the first study concerning such a noticeable number of cases of CMV resistance after valganciclovir therapy.

These patients met some critical risk factors for the emergence of CMV resistance. Indeed, all of them were high-risk patients for CMV infection with CMV D^+/R^- serostatus for all SOT recipients and D^-/R^+ serostatus for all SCT recipients. At the time of CMV resistance testing (i.e., CMV resistance suspicion), the level of viral replication was high since the median CMV load in blood of the patients was 4.7 log. Moreover, in patients who received intravenous curative treatments with ganciclovir +/− foscarnet prior

Table 1
Patients characteristics, CMV infection/disease, and treatments with oral valganciclovir.

Pt	Gender	Age (years)	Tx	CMV antibody status (D/R)	Number of blood samples[a]	Onset of CMV infection from Tx (days)	Symptomatic CMV infection	Valganciclovir treatment			Outcome
								Type[b]	Dose (q.d.)	Start and stop dates from Tx (days)[c]	
1	M	67	Heart	+/−	13	68	CMV syndrome	Prophylactic	900 mg	0 to 90	Alive
2	M	17	Heart	+/−	12	118	None	Prophylactic	900 mg	0 to 90	Alive
3	M	50	Kidney + liver	+/−	27	82	CMV syndrome	Prophylactic	450 mg[d]	0 to 90	Alive
4	M	46	Heart	+/−	15	31	Oesophagitis	Preemptive	900 mg	31 to 104 39 to 72	Alive
5	M	52	Heart	+/−	20	39	None	Preemptive	900 mg	79 to 129 154 to 255	Alive
6	M	22	Heart	+/−	13	41	None	Preemptive	900 mg	48 to 195	Alive
7	F	42	Kidney	+/−	41	30	CMV syndrome	Maintenance	450 to 900 mg[d]	51 to 71 107 to 150	Alive
8	M	36	Lung	+/−	16	55	CMV syndrome	Maintenance	900 mg	70 to 125	Alive
9	F	39	Stem cell	−/+	18	28	Pneumonia	Maintenance	450 to 900 mg[d]	107 to 227	Dead[e]
10	M	51	Stem cell	−/+	22	29	None	Maintenance	900 mg	72 to 115	Alive
11	F	60	Stem cell	−/+	48	13	Colitis	Maintenance	900 mg	155 to 212	Alive

D, donor; Pt, patient; R, recipient; Tx, transplantation.

[a] Number of blood samples collected for the patient from the time of transplantation until the time of CMV resistance detection.

[b] Maintenance treatment with valganciclovir was initiated after intravenous curative treatment with ganciclovir (#7, #8 and #10) or ganciclovir and foscarnet (#9 and #11).

[c] Valganciclovir treatment was discontinued for patients #5 and #7 (3 and 2 successive treatments, respectively).

[d] Dose adjustment of valganciclovir according to renal function.

[e] Multiple organ failure on day 241 posttransplantation.

Table 2
Primers used for UL97 and UL54 gene amplification and sequencing.

Target gene	Function	Name	Sequence (5′ → 3′)
UL97	First-round PCR (outer primers)	UL97PCR1S	F: GTGCTCACGGTCTGGATGT
		UL97PCR1AS	R: CGGTGGGTTTGTACCTTCTC
UL97	Second-round PCR (inner primers) and sequence reaction	UL97PCR2S	F: GCACAACGTCACGGTACATC
		UL97PCR2AS	R: ACCTTCTCTGTTGCCTTTCC
UL54	First-round PCR (outer primers)	UL54-1	F: ATCTGCTGGAGCAGGGTTTT
		UL54-11	R: CCAATCGCTTAATGACGGCA
UL54	Second-round PCR (inner primers)	UL54-2	R: TTGACGGTACAGCGAGATGT
		UL54-3	F: GCGTCGACTTGTGATATCGA
UL54	Sequence reaction	UL54-4	R: ATCCTCAAAGAGCAGCGAGA
		UL54-5	F: GCGCGGTTCATCAAAGACAA
		UL54-6	R: AAAGCGGACAAACACGCTGT
		UL54-7	F: TGGCTAAAATTCCGTTGCGG
		UL54-8	R: ACCTTTGCTGTAGTGGTTGG
		UL54-9	R: GCATACAGGGTACATGTCGAT
		UL54-10	F: TCGGCTTCTCACAACAATC
		+UL54-2 and UL54-3	

F, forward primer; R, reverse primer.

to maintenance treatment with valganciclovir, CMV load was still detectable (2.5–3.3 log) in 3 out of 5 patients when curative therapy was interrupted and valganciclovir was initiated at a maintenance dose. Thus, the continuous viral replication under this maintenance dose of antiviral might have constituted a risk factor for the emergence of resistance. Among SOT recipients receiving valganciclovir prophylaxis, the emergence of drug-resistant CMV infection occurred during the prophylactic treatment in 2 patients. Regarding the third one (patient #2), the CMV load reached the threshold of quantification of our technique (1.4 log) several times during the 90-day prophylaxis period before it became higher on day 118 (data not shown). This indicated that the virus was able to replicate despite the antiviral pressure, leading to the emergence of a resistant strain at the end of prophylaxis. These two latter points underline the need to use a very sensitive method to detect CMV DNA, in particular by using whole blood instead of plasma (Razonable et al., 2002). In the VICTOR study, which compared oral valganciclovir and intravenous ganciclovir for the treatment of CMV disease in SOT recipients, more than 50% of the patients who received valganciclovir maintenance treatment after a 3-week induction treatment with either intravenous ganciclovir or oral valganciclovir had a CMV viremia still detectable at the time of the shift (Asberg et al., 2007). Data concerning the long-term follow-up of those patients would be useful in order to investigate the potential emergence of CMV resistance in this context. An interim analysis in a subgroup of patients from this study has already reported some cases of CMV resistance to ganciclovir (Boivin et al., 2007).

For 10 patients, ganciclovir resistance detected after valganciclovir therapy was associated with one single mutation lying in UL97 phosphotransferase (Chou, 2008; Ijichi et al., 2002). These amino acid changes were mapped to codons 460 and 592–603, as previously reported for clinical isolates resistant to ganciclovir (Chou et al., 2002). Moreover, these results fit in with the pattern of resistance previously reported in AIDS patients treated with valganciclovir for CMV retinitis (Boivin et al., 2001). Thus, the type of immunosuppression (HIV infection or transplantation) does not seem to influence the resistance pattern of CMV following valganciclovir therapy. Unlike other patients, two distinct resistance mutations were observed for the patient #6: one in UL97 phosphotransferase (E596G), conferring resistance to ganciclovir, and one in UL54 DNA polymerase (L545S), conferring cross-resistance to ganciclovir and cidofovir (Cherrington et al., 1998; Cihlar et al., 1998). The selection of 2 different mutations in both viral genes was likely related to the prolonged and continued preemptive valganciclovir treatment (147 days). Of note, none of the 4 resistance mutations of CMV after valganciclovir therapy previously reported in transplant recipients (T659I, del 601–603, M460V in UL97 phosphotransferase; D413A in DNA polymerase) was identified in our study (Humar et al., 2005; Marfori et al., 2007).

Concerning the mutations associated with the natural polymorphism of viral enzymes, several mutations previously reported have been observed in the DNA polymerase (Chou et al., 1999; Fillet et al., 2004). In UL97 phosphotransferase, the mutation N510S has been detected in only one patient (#6). It has been previously described in CMV clinical isolates susceptible to ganciclovir (Erice et al., 1998; Lurain et al., 2001). Nevertheless, this mutation has been found also in a CMV isolate resistant to ganciclovir with a deletion of UL97 codons 591–594 (Chou et al., 1995). In our study, this mutation was detected in a CMV isolate resistant to both ganciclovir and cidofovir. Thus, the exact role of this mutation, natural polymorphism or compensatory mutation, remains unclear and needs further investigation.

Moreover, 6 novel mutations of unknown significance in UL97 and UL54 genes have been identified. The change V498I in UL97 phosphotransferase is located between the two conserved regions VII and IX of the protein. Similarly, the 5 novel mutations in UL54 DNA polymerase lie within nonconserved regions of the protein. In particular, 4 of them are located between domains delta-C and II (P608S, T610M, G629S) and between domains III and I (S880L), where a natural polymorphism of the enzyme has been previously reported (Fillet et al., 2004). The last amino acid change E315D is located between the N-terminal region and domain IV. Because of their location outside the conserved domains, all these mutations are likely considered to be associated with the natural polymorphism of viral proteins, rather than antiviral resistance. Nevertheless, further studies are required to ascertain the true nature of these novel mutations.

The outcome of patients suffering CMV disease associated with drug-resistant virus is generally poor (Limaye, 2002). In our study, ganciclovir resistance of CMV was not associated with a poor outcome since the cause of death for the only patient who died could not be directly related to CMV. The recent systemic analysis performed by Sun et al. (2008) concerning data from studies using valganciclovir as preemptive therapy or prophylaxis for CMV in SOT recipients evidenced that patients in the preemptive group developed no late-onset CMV disease (>90 days posttransplantation). In our study, the patients receiving valganciclovir preemptive therapy (#4, #5, and #6) are consistent with this since only one

Table 3
CMV UL97 phosphotransferase and UL54 DNA polymerase mutations.

Pt	CMV load (log[copies/mL blood]) at the time of		Time of CMV resistance testing after Tx (days)	UL97 phosphotransferase mutations			UL54 DNA polymerase mutations		
	Valganciclovir starting	CMV resistance testing		Resistance mutations	Polymorphism mutations	Novel mutations	Resistance mutations	Polymorphism mutations	Novel mutations
1	BLQ	4.9	145	A594V	No	No	No	S655L N685S A885T N898D	P608S
2	BLQ	3.7	139	M460I	No	No	No	A885T S897L	No
3	BLQ	3.7	126	C603W	No	No	No	S655L N685S A885T N898D	G629S
4	4.7	5.7	114	A594P	No	No	No	S655L N685S A885T N898D	No
5	3.0	4.7	255	C603W	No	No	No	A885T S897L	T610M
6	4.5	3.2	228	E596G	N510S	No	L545S	S655L N685S A693T A885T N898D	E315D
7	3.1	4.0	150	A594T	No	V498I	No	S655L F669L N685S A885T N898D	No
8	BLQ	4.8	111	L595S	No	No	No	S655L N685S G874R A885T L890F N898D	No
9	3.3	5.1	227	L595S	No	No	No	A885T P887S N898D	No
10	BLQ	4.9	117	L595S	No	No	No	V355A N685S A688V A885T	S880L
11	2.5	3.1	214	C592G	No	No	No	A885T S897L	No

BLQ, below the level of quantification (<1.4 log); Pt, patient; Tx, transplantation.

of them developed an early-onset CMV disease (≤90 days post-transplantation). For the management of CMV resistance, 8 patients received intravenous foscarnet, considered as the antiviral of choice in the case of ganciclovir resistance, and 3 patients received no more antiviral treatment although they ultimately displayed a good control of CMV load.

The observations in this study, in addition to the cases already reported in the literature (Eid et al., 2008; Humar et al., 2005; Marfori et al., 2007), suggest that the emergence of CMV resistance after valganciclovir therapy is not uncommon. Since valganciclovir tends to be more and more used in transplant recipients, the incidence of CMV resistance has to be closely monitored. Moreover, the results presented here highlight the differences regarding the use of valganciclovir among the different transplantation departments. Clinical practice has already shifted towards outpatient oral therapy. Obviously, a multicentric study is needed to identify the different pathways of valganciclovir treatments (strategies, doses, durations), to define the best use of this molecule in transplant recipients in order to avoid the emergence of CMV resistance, and to evaluate the time of emergence of CMV resistance after the start of valganciclovir according to the treatment strategy. Despite the small number of patients reported to date and the absence of a controlled study, the possibility of CMV resistance following valganciclovir use should be taken into consideration in particular among high-risk patients for CMV infection. Further studies are warranted to clarify this question. Nevertheless, as a first step, possible immediate actions might be discussed: (i) to perform monitoring of CMV infection (about once a month) during prophylactic treatments, (ii) to start preemptive treatments before CMV load becomes too high (a proposed threshold could be 3.0 log in whole blood) in order to increase the likelihood of CMV eradication prior to the potential emergence of resistance, and (iii) to assess the efficacy of intravenous curative therapies as the total eradication of CMV (i.e., a negative result using a sensitive real-time PCR method on whole blood) before the start of maintenance treatment.

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