Selection of the same mutation in the U69 protein kinase gene of human herpesvirus-6 after prolonged exposure to ganciclovir in vitro and in vivo

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After serial passage in the presence of increasing concentrations of ganciclovir (GCV) in vitro, a human herpesvirus-6 (HHV-6) mutant exhibiting a decreased sensitivity to the drug was isolated. Analysis of drug susceptibility showed that the IC50 of this mutant was 24-, 52- and 3-fold higher than that of the wild-type (wt) IC50 in the case of GCV, cidofovir and foscarnet, respectively. Genotypic analysis showed two single nucleotide changes as compared to the wild-type: an A → G substitution of the U69 protein kinase (PK) gene resulted in an M318V amino acid substitution and the other change, located in the C-terminal part of the U38 gene, resulted in an A961V amino acid substitution within the DNA polymerase. The M318V change was located within the consensus sequence DISPMN of the putative catalytic domain VI of the PK. This change was homologous to the M460V and M460I changes that had been reported previously within the consensus sequence DITPMN of the human cytomegalovirus (HCMV) UL97 PK and associated with the resistance of HCMV to GCV. The M318V change was also detected by PCR in HHV-6-infected PBMCs from an AIDS patient who had been treated with GCV for a long period of time and exhibited a clinically GCV-resistant HCMV infection. These findings provide strong circumstantial evidence that the M318V change of the PK gene is associated with resistance to GCV and raise the question of cross resistance to this drug among different betaherpesviruses.

Introduction

Human herpesvirus-6 (HHV-6) was isolated initially from lymphocytes of patients with AIDS and lymphoproliferative disorders (Salahuddin et al., 1986). The genome of HHV-6 (U1102 strain) was sequenced entirely (Gompels et al., 1995) and compared to that of human cytomegalovirus (HCMV) (Lawrence et al., 1990). Sequence analysis indicated an overall sequence similarity of 66%, which confirmed the classification of both viruses within the same Betaherpesvirinae subfamily. Two genetically distinct variants (A and B) were described and the U1102 strain was classified as HHV-6 variant A (Ablashi et al., 1991; Aubin et al., 1992; Schirmer et al., 1991). Recently, in the case of the Z29 and HST strains, the complete genome sequence of HHV-6 variant B has been determined. Comparing the sequences of HHV-6 variants A (U1102 strain) and B has shown 90% nucleotide sequence similarity and 94% amino acid identity (Dominguez et al., 1999; Isegawa et al., 1999).

HHV-6 is the causative agent of exanthem subitum in young children (Yamanishi et al., 1988). Because of its tropism for T lymphocytes, HHV-6 is thought to act as a co-factor of human immunodeficiency virus (HIV) in AIDS progression, but this hypothesis is still being debated (Ablashi et al., 1998; Garzino-Demo et al., 1996; Lusso & Gallo, 1995). HHV-6 has been also linked to several other diseases, such as multiple sclerosis, chronic fatigue syndrome and tumours (Ablashi et al., 2000; Berti et al., 2000; Ongrati et al., 1999), but all of these results remain controversial (Dorrucci et al., 1999; Taus et al., 2000). There are now convincing studies that indicate that HHV-6 behaves as an opportunistic pathogen in liver, renal and bone marrow transplant recipients (Humar et al., 2000; Ratnamohan et al., 1998; Rogers et al., 2000). Now, the precise frequency of such HHV-6-induced diseases has to be evaluated. Although the spectrum of HHV-6 pathogenicity...
still needs to be clarified, it has become clear that clinically serious HHV-6 infections should benefit from specific antiviral therapy, as pointed out in several reports (Cole et al., 1998; Mookerjee & Vogelsang, 1997).

In vitro, HHV-6 susceptibility to antiviral compounds was found to be broadly similar to that of HCMV. HHV-6 is sensitive to ganciclovir (GCV), a nucleoside analogue, cidofovir (CDV or HPMPC), a nucleoside phosphonate analogue, and foscamet (PFA), a pyrophosphate analogue, which are the major antiviral agents used currently to treat HCMV infections (Biron et al., 1985; Crumpacker, 1996; Freitas et al., 1985). HHV-6 is relatively resistant to acyclovir (ACV). It is known that suboptimal, long-term therapy of HCMV infection leads to the emergence of drug-resistant HCMV. Numerous studies have shown that nucleotide mutations in two genes, the DNA polymerase (pol) (UL54) gene and the protein kinase (PK) (UL97) gene, are involved in this resistance (Baldanti et al., 1996; Chou et al., 1995; Erice, 1999; Lurain et al., 1994; Sullivan et al., 1993). Pol is the final target of the three major antiviral agents and many of the mutations in the UL54 gene induce cross resistance to two or three drugs. The biological role of PK, which is encoded by the UL97 gene and which is also known as GCV kinase, is not understood completely, but PK has been shown to convert GCV and ACV into their monophosphate derivatives. Accordingly, mutations in the UL97 gene induce GCV resistance, but the HCMV strains carrying these mutations remain susceptible to HPMPC and PFA, unless they also carry UL54 gene mutations.

HHV-6 infection has been found to be frequently associated with HCMV infection and is probably underestimated in this context as it is not generally the target of specific diagnostic procedures. Therefore, it may be reasonably assumed that HHV-6 is exposed to anti-HCMV drugs in the context of therapies driven both by virological HCMV markers and by clinical symptoms of the HCMV disease. This unrecognized exposure to drugs is susceptible to significantly inhibit HHV-6 replication, but, alternatively, may lead to the selection of drug-resistant HHV-6 strains. To date, there is no available data on HHV-6 resistance to GCV or on the possible molecular mechanisms that sustain this process. As with HCMV, HHV-6 pol, which is encoded by the U38 gene, is thought to be the common target of antiviral drugs. It has been shown that HHV-6 PK, which is encoded by the U69 gene and is homologous to the HCMV UL97 gene product, can phosphorylate GCV and confers GCV sensitivity to baculovirus grown in insect cells expressing HHV-6 PK (Ansari & Emery, 1999). However, there continue to be questions concerning the role of HHV-6 PK in HHV-6-infected human cells as well as the existence of specific U69 gene mutations that induce GCV resistance.

We decided to address these questions using two distinct approaches: (i) the selection and characterization of GCV-resistant HHV-6 isolates in vitro using prolonged exposure to GCV; and (ii) the characterization of HHV-6 isolates present in vivo in HCMV-infected patients treated with GCV for a long period of time. The results presented here converge to suggest that a U69 gene mutation induces the resistance of HHV-6 to GCV.

### Methods

#### Laboratory virus strains and cells.

- The origin of the three HHV-6 variant A strains (SIE, TAN and Ul1102) and the seven HHV-6 variant B strains (BLA, BLE, BOB, BOU, MAR, MBE and TRA) studied have been described previously (Agut et al., 1988, 1989; Aubin et al., 1992). These virus strains were propagated in PBMCs from healthy donors as reported previously (Agut et al., 1989). The HST strain, a HHV-6 variant B isolated from a patient with exanthem subitum (Yamanishi et al., 1986), was kindly provided by Koichi Yamanishi (Osaka University Medical School, Osaka, Japan). The HST strain was adapted to grow in the MT4 cell line, which is derived from a patient with adult T-cell leukaemia and is human T-lymphotropic virus type I-transformed (Manichanh et al., 2000). These cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated foetal calf serum in the presence of 1% heat-inactivated foetal calf serum in the presence of 20 µg/ml amikacin and vancomycin and 29 µg/ml l-glutamine. HHV-6 infections were carried out at a concentration of 106 cells/ml and at an m.o.i. of 0.01 TCID50 per cell, starting from virus stocks that had been preliminary titrated on the same cells by end-point dilution assays.

#### Patient samples.

- Heparinized blood samples were obtained from 21 AIDS patients with HCMV disease. Each patient was followed up at the Pitie-Salpetriere Hospital in Paris. The 21 patients were treated with GCV either alone or in combination with PFA and CDV. In addition, one blood sample was obtained from one healthy untreated HHV-6-seropositive volunteer. PBMCs were separated from whole blood by centrifugation on Ficoll–Hypaque (Pharmacia), washed with PBS and stored at −80 °C until use.

#### Antiviral agents and monoclonal antibody (MAB).

- GCV, the nucleoside analogue 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (Roche) was used for the selection of GCV-resistant HHV-6 mutants and susceptibility assays. Two other antiviral compounds, CDV/HPMPC, (5)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine (Pharmacia & Upjohn), and PFA (Astra), were evaluated for their activity against HHV-6 wild-type (wt) virus and the GCV-resistant mutant. MAb 7C7 (Argene Biosoft) was used for the detection of HHV-6 antigen expression by means of immunofluorescence assays (IFA) and flow cytometry in the follow-up of HHV-6 infection and susceptibility assays, respectively (Manichanh et al., 2000; Robert et al., 1998).

#### Antiviral drug susceptibility assays.

- Drug sensitivities were determined as described previously (Manichanh et al., 2000). Briefly, MT4 cells were infected at an m.o.i. of 0.01 TCID50 per cell during 1 h of incubation at 37 °C in the presence of 5% CO2. Cells were recovered by centrifugation, resuspended in culture medium and distributed at the concentration of 2 x 105 cells per well in a 24-well plate (Costar) in a 50% of the presence of the appropriate concentrations of the drug. Two wells containing infected cells and two containing mock-infected cells without the drug were included as controls. At day 8 post-infection, virus antigen expression was analysed by flow cytometry using MAb 7C7. IC50 values were calculated as described previously (Manichanh et al., 2000).

#### DNA extraction.

- PBMCs or infected MT4 cells were resuspended in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, pH 8.0) containing 1% SDS and 200 µg/ml proteinase K. After 2 h of incubation at 56 °C, total DNA was extracted with an equal volume of TE-saturated
Resistance of HHV-6 to ganciclovir

Table 1. Primers used to amplify and sequence HHV-6-specific products

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Sequence (5’ → 3’)</th>
<th>Orientation</th>
<th>Location</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCVKA</td>
<td>U69</td>
<td>CGACGAAATAATTGATACGCGACGC</td>
<td>+</td>
<td>103776–103799*</td>
<td>PCR, sequencing</td>
</tr>
<tr>
<td>GCVKA1</td>
<td>U69</td>
<td>ATGAAACTGTCATGATGCC</td>
<td>+</td>
<td>105320–105337</td>
<td>Sequencing</td>
</tr>
<tr>
<td>GCVKA2 (HHV-6 variant A)</td>
<td>U69</td>
<td>AAGCTGTAACAAATTCCTGTG</td>
<td>+</td>
<td>104958–104976*</td>
<td>Sequencing</td>
</tr>
<tr>
<td>GCVKA2 (HHV-6 variant B)</td>
<td>U69</td>
<td>CAAATCCCGTTGATTGAGATGC</td>
<td>+</td>
<td>105768–105789</td>
<td>Sequencing</td>
</tr>
<tr>
<td>GCVKA3</td>
<td>U69</td>
<td>TGTTCAATATGCGCGAGGC</td>
<td>+</td>
<td>106161–106179</td>
<td>Sequencing</td>
</tr>
<tr>
<td>GCVKB</td>
<td>U69</td>
<td>CGGCCACAGAACTCATGTTTGCAG</td>
<td>+</td>
<td>105580–105604*</td>
<td>PCR, sequencing</td>
</tr>
<tr>
<td>GCVKB1 (HHV-6 variant A)</td>
<td>U69</td>
<td>CAACCTGGGAAAAAGGC</td>
<td>–</td>
<td>105091–105108*</td>
<td>Sequencing</td>
</tr>
<tr>
<td>GCVKB1 (HHV-6 variant B)</td>
<td>U69</td>
<td>CGACCTGAGCAAAGCAC</td>
<td>–</td>
<td>106230–106246</td>
<td>Sequencing</td>
</tr>
<tr>
<td>GCVKB2</td>
<td>U69</td>
<td>CCGAAGACTCGAGCCATAG</td>
<td>–</td>
<td>105589–105600</td>
<td>Sequencing</td>
</tr>
<tr>
<td>POLA</td>
<td>U38</td>
<td>TAGACAGGATCTAGTTAGAG</td>
<td>–</td>
<td>59619–59640*</td>
<td>PCR, sequencing</td>
</tr>
<tr>
<td>POLABIS</td>
<td>U38</td>
<td>CAGAGTATAGGTTTTAAGAG</td>
<td>–</td>
<td>60554–60574</td>
<td>Sequencing</td>
</tr>
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<td>U38</td>
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<td>–</td>
<td>60055–60073</td>
<td>Sequencing</td>
</tr>
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<td>U38</td>
<td>GACCCGCTGATATATATG</td>
<td>–</td>
<td>59534–59553</td>
<td>Sequencing</td>
</tr>
<tr>
<td>POLA3</td>
<td>U38</td>
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<td>Sequencing</td>
</tr>
<tr>
<td>POLA4</td>
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<td>GCATCTTATGCTGTCAG</td>
<td>–</td>
<td>58297–59317</td>
<td>Sequencing</td>
</tr>
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<td>POLA5</td>
<td>U38</td>
<td>TGAGATTTCACATAGACAC</td>
<td>–</td>
<td>58797–58818</td>
<td>Sequencing</td>
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<tr>
<td>POLA-MOR</td>
<td>U38</td>
<td>CAGACGCCATAAATGATAGACGGC</td>
<td>–</td>
<td>57706–57728</td>
<td>PCR, sequencing</td>
</tr>
<tr>
<td>POLB</td>
<td>U38</td>
<td>CGTGTACAAAAGCACAAACCT</td>
<td>+</td>
<td>56521–56541*</td>
<td>PCR, sequencing</td>
</tr>
<tr>
<td>POLB1</td>
<td>U38</td>
<td>CCGAAGAGCTCTTTCCTCAC</td>
<td>+</td>
<td>58691–58708</td>
<td>Sequencing</td>
</tr>
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<td>POLB2</td>
<td>U38</td>
<td>CTATAACAGAAGATATGCGGCC</td>
<td>+</td>
<td>58777–58797</td>
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</tr>
<tr>
<td>POLB3</td>
<td>U38</td>
<td>GTTAATCTATATCCTCC</td>
<td>+</td>
<td>59399–59416</td>
<td>Sequencing</td>
</tr>
<tr>
<td>POLB4</td>
<td>U38</td>
<td>GTTGGTCAATTTGCTTGACCATEC</td>
<td>+</td>
<td>59896–60006</td>
<td>Sequencing</td>
</tr>
<tr>
<td>POLB-MOR</td>
<td>U38</td>
<td>CATTAACCCTCTGCAATAGCC</td>
<td>+</td>
<td>57507–57527</td>
<td>PCR, sequencing</td>
</tr>
<tr>
<td>O10</td>
<td>U31</td>
<td>GATCCGCGCGCTACAAAACAC</td>
<td>–</td>
<td>46230–46249*</td>
<td>PCR</td>
</tr>
<tr>
<td>O15</td>
<td>U31</td>
<td>CGCGTTCACACAGCATGAACTCTC</td>
<td>–</td>
<td>45419–45442*</td>
<td>PCR</td>
</tr>
</tbody>
</table>

* Refers to the nucleotide sequence of the U1102 strain (Gompels et al., 1995).

Results

Isolation of a GCV-resistant HHV-6 mutant

MT4 cells were infected with the HST strain of HHV-6 and incubated in the presence of GCV at the initial concentration of


8 μM. The development of HHV-6 infection was specifically monitored by testing aliquot fractions of the cell culture twice a week. IFA was performed on cultured cells and end-point dilution PCR was carried out on the supernatant in parallel. When a positive IFA signal was observed in more than 50% of cells and/or a positive PCR signal was detected in the supernatant at a dilution of 10−4 or lower, the cell culture was divided into two equal fractions and naïve (i.e. not yet exposed to either GCV or HHV-6) MT4 cells were added at the ratio 2:1. This serial co-culture propagation was designed in order to try to avoid any selection of MT4 cells exhibiting a putative higher resistance to GCV-induced cytopathic effects. One resulting subculture was maintained at the same GCV concentration, while its counterpart was exposed to a 2-fold higher concentration of the drug. This procedure was repeated with every subsequent passage of the virus. When the replication of HHV-6 was not considered to be sufficient at a given concentration of GCV, additional passages were performed at the 2-fold lower concentration until the IFA and PCR end-points for HHV-6 replication were obtained. As controls, passages of HST in MT4 cells in the absence of GCV were performed in parallel to each step of selection under GCV pressure. After numerous serial passages over a period of 9 months, a reproducible efficient propagation of HHV-6 by coculture was obtained in the presence of 128 μM GCV. The putative GCVR1 HST strain was purified twice by end-point dilution in the presence of this drug concentration using culture supernatant as a virus stock. A stock of purified virus with an infectious titre of 106 TCID50/ml was ultimately obtained, designated GCVR1 HST and submitted to further characterization.

Phenotypic analysis of GCVR1 HST

In the absence of GCV, GCVR1 HST exhibited replication dynamics similar to that of wt virus in terms of antigen expression and virus yield, as assessed by IFA and PCR, respectively (data not shown). GCVR1 HST was subsequently tested by flow cytometry-based susceptibility assay against three antiviral compounds, GCV, CDV and PFA. The results obtained from two independent experiments, each involving duplicate assays for each drug concentration, demonstrated a decrease in the sensitivity of GCVR1 HST to the three drugs tested as compared to wt virus (Table 2). Comparison of the IC50 values indicated that GCVR1 HST was 24-, 52- and 3-fold less sensitive to GCV, CDV and PFA, respectively, than wt HST. Although it had been selected in vitro under GCV pressure only, GCVR1 HST was cross-resistant to CDV and, to a much lower extent, PFA.

Table 2. Sensitivity of the wt HST and GCVR1 HST strains to antiviral compounds

The index of resistance is defined as the ratio of IC50 (μM) of GCVR1 HST to wt HST.

<table>
<thead>
<tr>
<th>Compound</th>
<th>wt HST</th>
<th>GCVR1 HST</th>
<th>Resistance Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCV</td>
<td>9 ± 1</td>
<td>222 ± 13.5</td>
<td>24</td>
</tr>
<tr>
<td>CDV</td>
<td>6 ± 0.65</td>
<td>315 ± 65</td>
<td>52</td>
</tr>
<tr>
<td>PFA</td>
<td>25 ± 3.5</td>
<td>79 ± 0.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Genetic polymorphism of the U38 and U69 genes

The unexpected complex pattern of GCVR1 HST susceptibility to antiviral compounds raised the question of multiple mutations concerning both the pol and the PK genes. As a preliminary step, a consensus nucleotide sequence of these two genes was established, since this question had not been addressed previously. Three distinct strains of HHV-6 variant A and seven strains of HHV-6 variant B, which have never been exposed to GCV, were analysed in order to investigate the spontaneous genetic polymorphism of these two genes. Among the three HHV-6 variant A strains (SIE, U1102 and TAN) studied, the overall variability of the U38 gene was 1.08%, (33 of 3039 nt); the deduced overall variability of the pol sequence was 0.45%, (5 of 1013 aa). A consensus sequence was thus established for HHV-6 variant A pol (Fig. 1) from which SIE, U1102 and TAN pol diverged by 0.1, 0.9 and 0.03%, respectively. Among the seven HHV-6 variant B strains (BLA, BLE, BOB, BOU, MAR, MBE and TRA) studied, the overall variability of the U38 gene was 0.46% and that of pol was 0.3% (3 of 1013 aa). A consensus pol sequence was defined for HHV-6 variant B strains (Fig. 1), which diverged by less than 0.2% from individual HHV-6 variant B strain sequences and by 2.2% from the consensus HHV-6 variant A sequence. A majority of intervariant diverging residues (19 of 22) were not involved in intravariant genetic polymorphism, pointing out the existence of variant-specific residues at particular positions of the pol sequence. A similar strategy of analysis was applied to the U69 gene, 1689 bp in length encoding HHV-6 PK (563 aa in the case of HHV-6 variant B) (Fig. 2). The overall nucleotide variability was 0.47% among HHV-6 variant A strains and 0.11% among HHV-6 variant B strains, leading to an amino acid variability of 0.71% and 0.53%, respectively, for the PK gene. The two consensus HHV-6 variant A and B PK sequences diverged by 5.7%, while the divergence of each strain to the corresponding variant consensus sequence did not exceed 0.4% and 0.2% in the case of HHV-6 variant A and B strains, respectively. Of note, there is an insertion of one codon in the PK gene of HHV-6 variant B at position 75 as compared to the HHV-6 variant A PK gene. Again, the majority of intervariant diverging residues did not participate in intravariant variability.

All together, these data demonstrated that HHV-6 pol and
Resistance of HHV-6 to ganciclovir

Fig. 1. Variability of the pol sequence among HHV-6 strains. Nucleotide sequencing was carried out on DNA amplified from HHV-6-infected cell cultures. The consensus HHV-6 variant A sequence (HHV-6 A cs) was established from the pol sequences of SIE, U1102 and TAN strains. The consensus HHV-6 variant B sequence (HHV-6 B cs) was established from the pol sequences of BLA, BLE, BOB, BOU, MAR, MBE and TRA strains. Dashes indicate identical amino acid residues in HHV-6 A cs and HHV-6 B cs. The open and closed circles correspond to the positions of amino acid residues that vary between variants A and B, respectively. The arrow indicates the position of the amino acid residue specifically modified in the case of GCVR1 HST.

PK were highly conserved proteins, with a clear intervariant variability, founded on the existence of well-defined variant-specific polymorphism positions, but a low intravariant variability.

Genetic analysis of GCVR1 HST

The U38 and U69 genes of GCVR1 HST were sequenced and compared to those of wt HST that had been serially propagated in parallel to GCVR1 HST selection. Only two mutations were detected (Figs 1 and 2). A C → T nucleotide substitution at position 2882 of the U38 gene resulted in an A^31^V amino acid substitution in the pol gene. An A → G nucleotide substitution at position 952 of the U69 gene resulted in an M^31^V amino acid substitution in the PK gene. The sequences of wt HST fit perfectly with the HHV-6 variant B consensus sequences and none of the two mutations was located at any of the polymorphic sites detected among distinct GCV-sensitive HHV-6 strains. The consensus sequences of HHV-6 pol and PK were tentatively aligned with
the homologous sequences of HCMV (data not shown) in order to predict the possible influence of the two mutations on enzyme function. The $A^{651}V$ mutation appeared to be far distant from the predicted catalytic domains of pol (the nearest putative catalytic domains of HHV-6 pol were domains VII and V at positions 771 and 803, respectively) and did not fit the position of HCMV mutations that had been involved in the resistance of HCMV to GCV (see Discussion). In contrast, the $M^{218}V$ mutation was located within the putative domain VI of HHV-6 variant B PK and affected the consensus sequence $D^{561}SPMN$, which is homologous to the HCMV $D^{560}ITPMN$ sequence. This sequence is believed to be a highly conserved region among protein kinases and, in the case of HCMV, has been shown to be the location of GCV-resistance mutations. Two distinct GCV-resistant HCMV strains, R6HS (Lurain et al., 1994) and C9209 (Chou et al., 1995), have been found to exhibit $M^{218}V$ and $M^{218}I$ amino acid substitutions in HCMV PK, respectively. These changes were both responsible for the GCV resistance phenotype and are homologous to the $M^{218}V$ change in HHV-6 PK. The results of the genetic analysis of GCVR1 HST thus strongly suggested that the $M^{218}V$ mutation of the HHV-6 PK was, at least in part, responsible for the phenotypic GCV resistance observed. However, a role of the $A^{651}V$ pol mutation for this phenotype, in the context of a single mutation-induced cross resistance to GCV, CDV and PFA, might also be considered.

**In vivo genetic analysis of HHV-6 variant B among GCV-treated patients**

In order to demonstrate unambiguously the causative role of the $M^{218}V$ PK mutation in GCV resistance, two distinct approaches, both of which were based on HHV-6 variant B replication in MT4 cells, were attempted: marker rescue experiments using the transfection of plasmids carrying the mutated gene and measurement of GCV phosphorylation rates with the hope of detecting a significant decrease of this activity in the presence of the mutation. To date, despite numerous attempts, both strategies have failed, in particular due to the difficulties of achieving a high multiplicity of infection and obtaining recombination events in MT4 cells (data not shown). We then turned to an alternative approach consisting of the study of the U69 gene among subjects exposed to GCV treatment for a long period of time and who were thought to suffer from a GCV-resistant HCMV infection. PBMC samples were obtained from 21 AIDS patients fulfilling these criteria and one untreated, healthy subject as a control. Five of the patients (24%) and the control subject were found to be HHV-6 infected.
Table 3. Characteristics of the studied HHV-6-positive subjects at the time of PBMC sampling

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>HIV infection status</th>
<th>HCMV infection status</th>
<th>Ongoing GCV therapy</th>
<th>Detection of specific HHV-6 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 M</td>
<td>22</td>
<td>AIDS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND, –</td>
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<tr>
<td>#2 M</td>
<td>46</td>
<td>AIDS</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND, –</td>
</tr>
<tr>
<td>#3 M</td>
<td>46</td>
<td>AIDS</td>
<td>+</td>
<td>Retinitis (ongoing)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>#4 M</td>
<td>42</td>
<td>AIDS</td>
<td>+</td>
<td>Retinitis (recovery)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>#5 M</td>
<td>34</td>
<td>AIDS</td>
<td>+</td>
<td>Retinitis (recovery)</td>
<td>+</td>
<td>ND, –</td>
</tr>
<tr>
<td>Control</td>
<td>F</td>
<td>33</td>
<td>No infection</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

ND, Not determined due to the lack of sufficient PBMC DNA.

6 variant B-positive by means of diagnostic PCR (Table 3). Due to the low virus load, genetic study of the U69 gene from these subjects required separate amplification of three overlapping U69 subfragments and their cloning prior to nucleotide sequencing. One AIDS patient, designated patient #4, exhibited the M$^{318V}$ mutation of PK, while the other five patients and the control subject did not. These findings were reproducibly obtained from two independent amplification runs from the same sample. Partial sequencing of the HHV-6 variant B pol gene indicated that the A$^{961V}$ mutation was absent in the PBMC sample from patient #4. Of note is the fact that, at the time of PBMC sampling, patient #4 had experienced a long-term exposure to GCV and exhibited clinical symptoms of ongoing HCMV retinitis despite the prolonged GCV therapy. These circumstances suggest the selection of the M$^{318V}$ mutation in vivo, in parallel to prolonged inefficient GCV therapy. Isolation of the corresponding HHV-6 variant B strain from PBMC culture was not possible and, thus, did not permit us to test its susceptibility to GCV. A second sample from patient #4 was obtained a few weeks later. The patient was beginning to recover from retinitis and was found to be HHV-6-negative by PCR. Thus, we could not confirm the persistence of the M$^{318V}$ mutation.

Discussion

This is, to our knowledge, the first report of HHV-6 resistance to GCV. The mutant GCVR1 HST displays a decreased susceptibility to GCV, CDV and, to a lesser extent, PFA. This phenotypic pattern is associated with the M$^{318V}$ change of HHV-6 PK, which was also selected in vivo after prolonged inefficient GCV therapy. The definitive proof for the causative role of the M$^{318V}$ amino acid substitution in GCV resistance is still lacking. However, there is strong circumstantial evidence of this causative role based on the following arguments: (i) the corresponding U69 gene mutation has not been implicated as a marker of genetic polymorphism among GCV-sensitive HHV-6 strains; (ii) this mutation is homologous to the mutations of the UL97 gene implicated in the GCV resistance of HCMV; (iii) this mutation is located at a predicted crucial site of HHV-6 PK, which corresponds to a canonic motif present in other protein kinases; (iv) the data in vivo and in vitro converge to demonstrate the presence of this mutation as HHV-6 variant B is able to replicate under usually efficient GCV concentrations.

HHV-6 resistance to antiviral compounds is still an emerging question. Although this virus has been found to be the cause of severe opportunistic infections among immunocompromised patients, there are, to date, only a few reports about the treatment of HHV-6 infection by GCV, PFA and CDV, either alone or in combination (Cole et al., 1998; Mookerjee & Vogelsang, 1997; Rieux et al., 1998). However, it is clear, in particular from our results, that HHV-6 is very often exposed to GCV in vivo through the prophylaxis and treatment of HCMV infections. Our findings indicate that this unrecognized exposure may select HHV-6 mutations distinct from the usual polymorphism profile of the virus. These mutations, in particular the reported M$^{318V}$ amino acid substitution, are likely to confer a replication advantage to HHV-6 mutants under GCV pressure. Due to the structural similarity of GCV and ACV, prolonged exposure to high concentrations of ACV, as in the situation of HCMV infection prophylaxis, might also be considered in terms of partial selective pressure against both HCMV and HHV-6 PKs. Although the IC$^{-50}$ of ACV against both viruses is high, we cannot rule out the hypothesis that partially effective ACV
concentrations may select PK changes, thus inducing a higher resistance profile to GCV. It should be analysed to what extent this unrecognized exposure of HHV-6 to antitherpetic compounds may induce the emergence of drug resistance and compromise the success of further treatments against this virus.

The mechanism of HHV-6 resistance to GCV remains to be understood. The function of HHV-6 PK needs to be clarified in this context. A role in the first step of GCV phosphorylation is strongly suggested by the GCV-induced cytopathic effect in insect cells expressing HHV-6 PK (Ansari & Emery, 1999). This mimics the effect observed with eukaryotic cells expressing herpes simplex virus (HSV) thymidine kinase and which are exposed to GCV, a well-known strategy for suicide gene therapy. The mutation M318V is believed to alter the phosphorylation activity of HHV-6 PK. This would consequently reduce the level of GCV monophosphate within the cell, leading to the decrease of final concentrations of GCV triphosphate active on HHV-6 pol. As mentioned previously, the proof of this deficient phosphorylating activity cannot be obtained under the current standard experimental conditions of HHV-6 infection and, therefore, requires other strategies.

However, another mechanism might be considered for HHV-6 resistance to GCV. As in the case of HCMV, mutations of the pol gene can induce GCV resistance, in particular by lowering the affinity of GCV triphosphate to the binding site of the enzyme and/or reducing the efficiency of the catalytic polymerization step involving this triphosphate analogue. In this case, the GCV-resistance pol gene mutations may also induce resistance to compounds that do not require the first step of phosphorylation into the monophosphate moiety to be active, e.g. CDV and PFA. GCVR1 HST does exhibit this pattern of cross resistance and, in this context, the role of the single pol A661V change must be discussed. This mutation is far distant from the predicted nucleotide binding and catalytic sites of HHV-6 pol, namely domains IV, II, VI, III, I and V located at positions 346, 551, 629, 662, 736 and 803, respectively (Teo et al., 1991). In particular, it is far distant from the domains that are homologous to those carrying GCV-resistance mutations in HCMV strains, domains IV and V (Lurain et al., 1992; Sullivan et al., 1993). Nevertheless, any amino acid change may alter the general conformation of the protein and, therefore, enzyme functions. As an example, two mutations of the HSV pol gene located at the positions 1007 and 1028 of the amino acid sequence, i.e. beyond the last conserved enzymatic domain, are able to induce resistance to HPMPA, a nucleotide analogue closely related to CDV (Andrei et al., 2000). However, in this case, the HPMPA-resistant mutants were not resistant to either ACV or PFA, suggesting that the mechanism of resistance was rather specific for HPMPA and did not affect the sensitivity to either nucleoside or pyrophosphate analogues. It is, therefore, tempting to assume that the A661V change of HHV-6 pol is responsible for its high resistance to CDV and moderate resistance to PFA. Its contribution to GCV resistance cannot be completely ruled out, in particular, because sequential processes may be considered for the emergence of GCV resistance: the U69 mutations generating low level resistance to the drug might be followed by the appearance of the U38 mutations inducing a higher level of resistance. This would be similar to the sequential emergence of the UL97 and UL54 mutations observed with GCV-resistant HCMV (Baldanti et al., 1996). However, when a retrospective analysis was carried out on the samples reserved from the serial intermediary passages under GCV, no temporal dissociation was observed in the emergence of either mutation in the GCV-resistant HHV-6 population. In any case, no mutation of HCMV analogous to A661V has been reported for GCV-resistant viruses and a unique role of A661V for the GCV-resistant phenotype, excluding any contribution of the M318V PK change, is unlikely. The question is why this mutation has been selected in vitro without any use of CDV during HHV-6 propagation and not in vivo in the HHV-6 variant B pol gene from patient #4. The GCV selection pressure in vitro broadly differs from that exerted in vivo due to the consistency of the drug concentration used, the monomorphic nature of cells sustaining virus replication and the absence of many other interfering factors specific for a human organism. In vitro, the selection of GCV-resistant HHV-6, which, in our hands, resulted from a long-standing procedure, may require some compensatory mutations in the pol gene that, by chance, may induce CDV resistance. This illustrates the fact, well known for other herpesviruses, that the isolation and characterization of drug-resistant HHV-6 mutants may be helpful to elucidate both the mechanisms of selective antiviral activity and the dynamics of genetic evolution under selective pressure.

We thank François Briaire and Christine Katlama for their help in the collection of PBMC samples, Koichi Yamanishi for kindly providing the HST strain of HHV-6 and Susan Orsoni for the critical reading of the manuscript. We are also grateful to Robert Snoeck, Lieve Naesens, Leen Debolle and Graciela Andrei for helpful discussions about herpesvirus resistance to antivirals. C.M. was sponsored by the Association Claude Bernard, Action Concertée Coordonnée des Sciences du Vivant, Association pour la Recherche sur le Cancer, MENRT (grant no. 98446).

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