Heterogeneity and Evolution of Thymidine Kinase and DNA Polymerase Mutants of Herpes Simplex Virus Type 1: Implications for Antiviral Therapy

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**Background.** Infections caused by acyclovir-resistant isolates of herpes simplex virus (HSV) after hematopoietic stem cell transplantation (HSCT) are an emerging concern. An understanding of the evolutionary aspects of HSV infection is crucial to the design of effective therapeutic and control strategies.

**Methods.** Eight sequential HSV-1 isolates were recovered from an HSCT patient who suffered from recurrent herpetic gingivostomatitis and was treated alternatively with acyclovir, ganciclovir, and foscavir. The diverse spectra and temporal changes of HSV drug resistance were determined phenotypically (drug-resistance profiling) and genotypically (sequencing of the viral thymidine kinase and DNA polymerase genes).

**Results.** Analysis of 60 clones recovered from the different isolates demonstrated that most of these isolates were heterogeneous mixtures of variants, indicating the simultaneous infection with different drug-resistant viruses. The phenotype/genotype of several clones associated with resistance to acyclovir and/or foscavir were identified. Two novel mutations (E798K and I922T) in the viral DNA polymerase could be linked to drug resistance.

**Conclusions.** The heterogeneity within the viral populations and the temporal changes of drug-resistant viruses found in this HSCT recipient were remarkable, showing a rapid evolution of HSV-1. Drug-resistance surveillance is highly recommended among immunocompromised patients to manage the clinical syndrome and to avoid the emergence of multidrug-resistant isolates.

**Keywords.** heterogeneity of HSV populations; drug-resistance dynamics in HSV; evolution of HSV populations; DNA polymerase; thymidine kinase; multiple HSV infections; hematopoietic stem cell transplantation (HSCT).

Diseases due to herpes simplex virus (HSV), although usually self-limited in the normal population, can be a major cause of mortality and morbidity in immunocompromised patients (ICs). Mucocutaneous HSV reactivation is very common among individuals with an impaired immune system, ranging from 15% among chronic lymphocytic leukemia patients receiving fludarabine to 90% among patients with acute leukemia or allogeneic hematopoietic stem cell transplantation (HSCT) [1].

Among ICs, HSV-associated disease may have an atypical appearance (more invasive disease, relatively slow healing, prolonged viral shedding, and risk of dissemination) and usually responds to acyclovir (ACV) treatment within a few days; ACV prophylaxis is highly efficacious in preventing relapses. Nevertheless, some of these infections become chronic and require prolonged antiviral treatment, favoring the emergence of drug-resistant viruses [2]. Among ICs, the prevalence...
rates of ACV-resistant (ACV\textsuperscript{r}) HSV varies from 3.5\% to 10.9\% compared with only 0.3\% to 0.7\% in immunocompetent individuals [3–6]. ACV resistance is caused by either mutations in the viral thymidine kinase (TK; necessary for activation of the drug) or DNA polymerase (pol; molecular target of ACV-triphosphate) genes [7–9]. The majority of the clinical ACV\textsuperscript{r} isolates have mutations in the viral TK gene [7].

Foscavir (PFA), a direct inhibitor of the virus DNA polymerases, is considered an alternative therapy for the treatment of ACV\textsuperscript{r} viruses due to alterations in the viral TK gene [10–12]. However, mutations in the viral DNA pol leading to PFA\textsuperscript{r} have also been reported. Most of the ACV\textsuperscript{r} strains that result from changes in the viral DNA pol gene are also PFA\textsuperscript{r} [13–15]. Furthermore, HSV drug-resistant mutants associated with alterations in both the TK and DNA pol genes have been described [16]. The acyclic nucleoside phosphonate (ANP) cidofovir (CDV, HPMPC, (S)-1-[3-hydroxy-2-(phosphonylmethoxypropyl)cytosine]) is independent of viral TK for its activation. It is considered an alternative therapy and has been shown efficacious in the treatment of ACV\textsuperscript{r} and/or PFA\textsuperscript{r} HSV infections [17–19].

Infections caused by ACV\textsuperscript{r} HSV have become a concern in several allogeneic stem cell transplantation centers because their reported incidence seems to be increasing, they cause major morbidity, and they can be life threatening [20], and their management is challenging [6, 21, 22]. An understanding of the evolutionary aspects of HSV infection is crucial to the design of effective therapeutic and control strategies. The purpose of this study was to investigate HSV dynamics by characterizing multiple temporal strains recovered from a patient with recurrent HSV type 1 (HSV-1) gingivostomatitis who also had leukemia and received HSCT. The present study reveals a rapid evolution of the viral population and the acquisition of multiple drug resistances due to simultaneous infection with strains bearing different mutations in the TK and/or DNA pol genes.

**MATERIALS AND METHODS**

**Patient and Clinical Specimens**

A 35-year-old woman was diagnosed with acute myelocytic leukemia M2 (karyotype 5q−) in April 2006. She was treated with Cytarabine and Idarubicin as induction chemotherapy. However, due to therapy failure, she received new induction chemotherapy with Cytarabine and Amsacrine that resulted in complete remission. She was given Cytarabine and Amsacrine as consolidation chemotherapy.

In October 2006, she underwent T-cell–depleted allogeneic transplantation with stem cells from her HLA-matched sister (5/8 HLA haploidentical) with alloreactive natural killer cells (Figure 1). The serological analyses of the donor (D) and recipient (R) were as follows: hepatitis A, Epstein-Barr, and varicella-zoster viruses: D+/R+; cytomegalovirus (CMV), hepatitis B and hepatitis C viruses: D−/R−; toxoplasma: D+/R−. Antibodies against HSV-1/HSV-2 were not tested before transplantation. The conditioning regimen consisted of Melphalan, Fludarabine, antithymocyte globulin, and total body irradiation. During the immediate posttransplant period, the patient received ciprofloxacin and ACV prophylactically (3× 400 mg by mouth or 3× intravenous 5 mg/kg daily). She did not develop any manifestation of graft-versus-host disease (GVHD) and left the hospital with a prophylactic treatment including ACV (400 mg 3 times per day), fluconazole, and sulfamethoxazole plus trimethoprim.

In December 2006, she received 2 doses of Rituximab (at days 60 and 67 posttransplantation) because of Epstein-Barr virus reactivation. In January 2007, she developed signs of GVHD and received corticosteroids and cyclosporine A. In March 2007, she presented with a herpetic gingivostomatitis under ACV treatment and then received PFA (90 mg/kg 2 times per day intravenously). She also had pneumonia due to *Streptococcus pneumonia* resistant to penicillin that responded to moxifloxacin (400 mg once daily given intravenously for 3 days and then orally for 7 days). At 167 days posttransplantation, she was hospitalized because of complete blindness, cough, and worsening of her general condition. The patient was not neutropenic at that time. However, due to a microangiopathy, cyclosporine was replaced by mycophenolate mofetil by mouth while corticosteroids were maintained. At the time of hospitalization, no HSV lesions were observed in the mouth, but ulcerative lesions developed quickly under prophylactic ACV. Considering the patient’s blindness, ganciclovir (GCV) was started to cover a possible cytomegalovirus (CMV) retinitis. An invasive pulmonary aspergillosis was diagnosed, and blindness was attributed to aspergillus endophthalmitis. Antifungal treatment (Voriconazole) was initiated; GCV was interrupted and replaced by PFA (90 mg/kg bid) at day 170 posttransplantation because of worsening of the mouth ulcerations. A cerebral nuclear magnetic resonance image identified several focal lesions, and treatment with pyrimethamine and clindamycin was started. This treatment was rapidly stopped; at day 178 posttransplantation, liposomal Amphotericin B was added because of progression of pulmonary aspergillosis noted in chest computed tomographic scan. Polymerase chain reaction amplification for HSV and CMV was negative in the cerebrospinal fluid, and several mouth rinses were performed because of the lack of improvement of the oral HSV lesions despite PFA treatment. In the meantime, the patient developed a new pneumococcal pneumonia complicated with bacteremia and received piperacillin–tazobactam (4 g 4 times per day). At day 200 posttransplantation, PFA was stopped and the patient received intravenous acyclovir (10 mg/kg 3 times per day). The HSV isolate recovered at that time showed ACV\textsuperscript{r} and PFA\textsuperscript{r}, and antiviral treatment was discontinued. The oral lesions remained stable, but mouth rinses and swabs remained positive for HSV-1. In the
following days, the patient’s general condition declined: the patient developed fever, pleural and pericardial effusion, hypothyroidism, tachycardia, and progressive respiratory failure. Despite active supportive treatment, the patient died 8 months after transplantation; no autopsy was performed (according to the will of the patient’s family).

**Cells and Viruses**

Human embryonic lung (HEL) fibroblasts (ATCC CCL-137) and Vero cells (ATCC CCL-81) were maintained in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 0.3% sodium bicarbonate. The HSV-1 reference strain Kos (ATCC VR-1493) was used. Stocks of clinical isolates were prepared in Vero cells. All viruses were obtained and used as approved by the Belgian institutional review board equivalent (Department Leefmilieu, Natuur en Energie, protocol SBB 219 2011/0011, and the Biosafety Committee KU Leuven).

**Plaque Purification of Clinical Samples**

Eight isolates were recovered from the patient (Figure 1). Each clinical isolate was then plaque-purified using standard procedures to obtain clones derived from a single virus [13, 14].

**Antiviral Assays**

Drug susceptibilities of different virus clones were determined (in the same conditions as for the reference HSV-1 strain) by virus cytopathic effect (CPE) reduction assays in HEL cells as previously described [14]. The 50% effective concentration (EC50) was defined as the drug concentration required to reduce the virus CPE by 50%.

**Viral TK and DNA pol Gene Sequencing**

To determine the genotype, sequencing of the entire TK and DNA pol genes of the different viral clones was performed as previously described [13, 23].

**RESULTS**

Phenotypic Analysis of Different Isolates

Antiviral resistance testing was performed on 8 viral isolates recovered from the HSCT recipient between 6 March and 19 June 2007 (Supplementary Table 1). Susceptibility profiles for several classes of compounds [nucleoside analogues (ACV, PCV, and GCV), pyrimidine analogues (BVDU), pyrophosphate analogues (PFA and PAA), and several ANPs including PME derivatives (PMEA, PMEDAP), PMEO-DAPy, HPMP]
derivatives (cidovir or HPMPC), HPMPO-DAPy, and HPMP-5-azaC were determined using a CPE reduction assay in HEL cells. The first HSV-1 isolate (70/2112) recovered from the patient at day 134 posttransplantation had a phenotype comparable to that of the reference laboratory strain Kos.

The other 7 isolates, which were recovered following consecutive therapy with ACV, GCV, and PFA between 11 May and 19 June, showed differences in the EC50 wild-type/EC50 clinical isolates ratios for TK-dependent drugs (ACV, GCV, PCV, and BVDU), pyrophosphate analogues (PFA and PAA), the PME derivatives (PMEA, PMEDAP), and PMEO-DAPy. None of the 7 isolates had significant differences in EC50 values (>2.5-fold) for HPMPC, HPMP-5-azaC, or HPMPO-DAPy.

**Genotypic Analysis of Different Plaque-Purified Viruses**

Plaque purification was performed, and several clones per isolate were selected in order to determine the isolates’ heterogeneity. Sequencing of the viral TK and DNA pol genes was performed for different clones (Table 1). Single viral populations could be identified for the first 2 isolates (70/2112 and 70/4888) under the present experimental conditions. Thus, all 6 clones isolated from the 70/2112 strain had a wild-type genotype, while all 11 clones recovered from the isolate 70/4888 harbored 1 mutation in the DNA pol (E798K) and another mutation in the viral TK (R176Q). All other isolates proved to be mixed populations of wild-type and mutant viruses (70/2112, 70/5293, and 70/6382) or mixed populations of different mutant viruses.

Three alterations in the viral TK were identified [the R176Q substitution and 2 frameshift mutations that resulted from a guanosine (G) insertion at nucleotides (Nts) 430–436 or of a G deletion at Nts 853–856]. The R176Q amino acid change in the TK was found alone (3 clones) or in combination with alterations in the viral DNA pol (20 clones). The frameshift mutations in the TK were found in combination with the DNA pol mutation I922T, except for 4 clones recovered from the 70/6382 isolate that had a G insertion at Nts 430–436 in the TK but no changes in the DNA pol. One clone recovered from the 70/5550 isolate bore the I922T amino acid change in the viral DNA pol and no alterations at the level of the viral TK. On the other hand, the DNA pol E798K change was always found in combination with the R176Q in the TK. In summary, 8 viral populations (1 wild-type and 7 drug-resistant) could be identified.

**Phenotypic Analysis of Different Mutant Viruses**

Next we phenotypically analyzed several clones representing the 7 types of mutant viruses (Figure 2). The EC50 values for these clones were compared with plaque-purified viruses derived from the first isolate (70/2112) recovered from the patient, which was considered the baseline isolate because it presented a wild-type phenotype and genotype (Table 1). In order to facilitate the visualization of the results, the log10 [EC50 mutant virus/EC50 wild-type] was also calculated (Figure 3).

Clones that only harbor a G insertion at Nts 430–436 in the viral TK showed a >2 log10 increase in EC50 values for ACV, BVDU, PCV, and GCV compared with wild-type clones, while no changes in sensitivity were observed for the pyrophosphate analogues and ANPs. A similar degree of resistance to TK-dependent drugs was noted for the clones bearing a G deletion at Nts 853–856.

Clones harboring the R176Q amino acid change in the TK had a different pattern of resistance compared with the TK frameshift mutants. In the case of the R176Q mutants, higher levels of resistance were noted for guanosine analogs (ACV, GCV, and PCV) than for the pyrophosphate analogs and ANPs. A similar degree of resistance to TK-dependent drugs was noted for the clones bearing a G deletion at Nts 853–856.

Although the DNA pol E798K change was not found singly, circumstantial evidence of the effect of the E798K mutation on drug resistance could be deduced by comparing the clones bearing the TK R176Q alone with those harboring the DNA pol E798K + TK R176Q mutations. Thus, the DNA pol E798K amino acid change was responsible for decreased sensitivity to the pyrophosphate analogs and to the PME derivatives.

<table>
<thead>
<tr>
<th>Isolate (date)</th>
<th>Number of Clones</th>
<th>Mutations in Viral</th>
<th>Thymidine Kinase</th>
<th>DNA pol</th>
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<tbody>
<tr>
<td>70/2112 (March 6)</td>
<td>6</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>70/4888 (May 11)</td>
<td>11</td>
<td>R176Q</td>
<td>E798K</td>
<td></td>
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<tr>
<td>70/5293 (May 15)</td>
<td>5</td>
<td>R176Q</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>70/5550 (May 23)</td>
<td>1</td>
<td>R176Q</td>
<td>—</td>
<td>I922T</td>
</tr>
<tr>
<td>70/5768 (May 29)</td>
<td>4</td>
<td>G ins. Nts 430–436 (frameshift)</td>
<td>I922T</td>
<td></td>
</tr>
<tr>
<td>70/5448 (May 19)</td>
<td>6</td>
<td>G ins. Nts 430–436 (frameshift)</td>
<td>I922T</td>
<td></td>
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<tr>
<td>70/6382 (June 19)</td>
<td>2</td>
<td>R176Q</td>
<td>—</td>
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<tr>
<td>70/5448 (May 19)</td>
<td>6</td>
<td>G ins. Nts 430–436 (frameshift)</td>
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<td>G ins. Nts 430–436 (frameshift)</td>
<td>I922T</td>
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Furthermore, the DNA pol E798K + TK R176Q afforded higher levels of resistance to ACV and PCV compared with the TK R176Q mutants. This is consistent with previous studies that demonstrated that different amino acid substitutions in the herpesvirus DNA pol result in cross-resistance between the pyrophosphate analogues and the PME derivatives, indicating that they have a common way of interacting with the viral enzyme [13, 24]. Additionally, the DNA pol E798K alteration had an impact on susceptibility to ACV and PCV, which is also in line with previously described DNA

Figure 2. Resistance properties of plaque-puriﬁed drug-resistant herpes simplex virus-type 1 clones bearing mutations in the viral thymidine kinase (TK) and/or DNA pol. Different plaque-puriﬁed viruses bearing speciﬁc mutations in the viral TK and/or DNA polymerase genes were compared with wild-type virus (plaque-puriﬁed viruses derived from the 70/2112 isolate) in the cytopathic effect reduction assays. At least 3 independent experiments were performed for each plaque-puriﬁed virus. Each individual point represents the mean values from independent experiments for 1 particular plaque-puriﬁed virus. The data are presented as a dot plot of the 50% effective concentration (EC50) for the drug-resistant clones vs the EC50 for the wild-type clones. Average (indicated as a horizontal line) ± standard errors (bars) are indicated for the different types of mutants and the wild-type clones. The sources of the compounds used in the antiviral assays were as follows: acyclovir [ACV, 9-(2-hydroxyethoxymethyl)guanine], GlaxoSmithKline, Stevenage, United Kingdom; the pyrophosphate analogues foscavir [PFA, foscarnet, phosphonoformate sodium salt] and phosphonoacetic acid [PAA], Sigma Chemicals, St Louis, MO; PMEA [adenefvir, ADV, 9-[2-(phosphonylmethoxyethyl)adenine]] and HPMPC [cidofovir, CDV, (S)-1-[3-hydroxy-2-(phosphonomethoxypropyl)cytosine]], Gilead Sciences, Foster City, CA; PMEDAP [9-[2-(phosphonomethoxy-ethyl)-2,6-diaminopurine], PMEPO-DAPy [6-[3-hydroxy-2-(phosphonooxypropoxy)-2,4-diaminopyrimidine], PMSO-DAPy [5-[3-hydroxy-2-(phosphonooxythoxyethyl)-2,4-diaminopyrimidine]], HPMPS-azaC [1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine], Dr Marcela Krecmerova, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic; brivudin [(E)-5-(2-bromovinyl)-1-(β-D-2'-deoxyribonanos-1-yl-uracil], BVDU, Searle, United Kingdom; ganciclovir [GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine, Roche, Basel, Switzerland; penciclovir [PCV, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine], Aventis, Frankfurt, Germany.
pol amino acid changes that result in ACV<sup>T</sup>, PCV<sup>T</sup>, and PFA<sup>T</sup> [7, 13].

The I922T substitution in the HSV-1 DNA pol had a much lower impact on drug resistance compared with the amino acid change E798K. Thus, when analyzing the data for the I922T mutation alone or in combination with different TK alterations, the I922T mutation was associated with changes in EC<sub>50</sub> compared with the wild-type virus of 2- (ACV), 3- (PFA and PAA), and 3- to 8- (PMEA derivatives and PMEO-DAPy) folds.

It should be noted that all the mutant viruses described here remained sensitive to HPMPC, HPMPO-DAPy, and HPMP-5-azaC (fold changes in EC<sub>50</sub> values ≤2.5 when comparing EC<sub>50</sub> of different mutant viruses with EC<sub>50</sub> of wild-type viruses).

Analysis of TK and DNA pol Mutations

Several amino acid substitutions in the HSV-1 TK are known to be linked to genetic polymorphism or interstrain variability (Figure 4). Six polymorphisms in the viral TK were identified in viruses recovered from the patient. Two types of frameshift mutations were observed in the isolates recovered from the patient. Among the 48 clones that had alterations in the viral TK, 23 clones presented the amino acid substitution R176Q, while 25 clones had a G insertion or a G deletion in homopolymeric runs leading to the production of a truncated protein (Table 1).

The R176Q change has been previously described in clinical isolates recovered from patients refractory to ACV [5, 25]. This mutation appears to confer a TK-altered phenotype because it was associated with higher levels of resistance to purine analogues compared with pyrimidine nucleoside analogues. This mutation appeared to have only local effects on the substrate-binding site, whereas the folding of the protein remained the same [26]. A significant decrease in the phosphorylating ability of the R176Q TK enzyme for acycloguanosine and to a much lesser degree for pyrimidine derivatives has been reported [27].

The amino acid substitutions in the HSV-1 DNA pol that are known to be linked to genetic polymorphisms or
associated with drug resistance (mostly located in the conserved regions) are indicated in Figure 5. In the present study, 2 known genetic polymorphisms and 2 novel amino acid changes (E798K and I922T) were identified in the viral DNA pol. The E798 and I922 residues are located in nonconserved regions of the viral enzyme; however, these amino acids are conserved at homologous positions among herpesviruses and are located in, respectively, the “fingers” and “palm” domains (Figure 6).

**DISCUSSION**

Systematic evaluation of the phenotyping and genotyping of sequential HSV-1 isolates in this patient revealed interesting features of the viruses that infected this individual. First, most of the viral isolates were heterogeneous mixtures of different types of drug-resistant viruses, in which the proportion of each viral variant determined the isolate’s phenotype and whether the patient was refractory to antiviral therapy.

Second, although DNA viruses are expected to have a low genetic variability, a rapid evolution of the viral population was noted with the appearance of 7 drug-resistant variants in a period of <3 months. Third, 2 novel amino acid changes in the viral DNA pol were associated with ACVr and PFAr, indicating that previously unrecognized mutations linked to drug resistance continue to be described. Our data point to the usefulness of multiple sampling and of analyzing the presence of HSV variants in sequential isolates to clearly determine their impact on clinical resistance. Typing drug resistance can then be useful for the adjustment of antiviral therapy.

It is well recognized that the HSV TK presents several homopolymeric nucleotide stretches (G or C) that function as hotspots for insertion or deletion of nucleotides (Figure 4). Approximately 50% of clinical and in vitro selected ACV TK mutants bear insertions or deletions in these homopolymeric regions, while the other 50% of mutants harbor single nucleotide substitutions in conserved and nonconserved regions of the TK gene [5, 6, 29]. It has been hypothesized that mutational
hot spots in the HSV TK could allow the emergence of TK-deficient isolates in recurrent lesions [30]. Also, homopolymers are the most abundant class of short sequence repeats (SSRs) in all viral genomes analyzed to date [31]. SSRs are probably a common property of large DNA viruses, being homopolymers across the viral genome mutational hot spots for evolutionary diversity in herpesviruses [31].

A high percentage of variants bearing alterations in the viral DNA pol could be detected among the 49 viral mutants. This can be explained by the fact that the patient received ACV, GCV, and PFA consecutively. It is difficult to determine which compound selected for the E798K and I922T mutation because the drug-resistant strains were recovered when antiviral treatment was halted. No treatment was administered to the patient at the moment that PFAr and ACVr were detected.

The genetic makeup of virus populations within a patient changes under the selective pressure of antiviral drugs. Interestingly, mutations occurred rather rapidly in this patient and were found in many of the plaque-purified isolates in different permutations. The question remains as to whether these mutations already existed within the patient or resulted from recombination events during the primary isolation process (which seems rather unlikely considering the very low recombination frequency among herpesviruses). Regardless, the different isolates had distinctive phenotypes and genotypes, illustrating how the virus can adapt to the selective drug pressure.

Although no mutant viruses were detected among the clones isolated from the first isolate (ie, 70/2112), we cannot exclude that minor amounts of resistant viruses were present in this isolate, nor can we exclude that the 70/4888 isolate did not contain very low levels of other mutant viruses. Deep sequencing should help in determining whether minor populations of resistant viruses were present in the different isolates.

Treatment of multidrug-resistant HSV infections represents a challenge for clinicians. Therapies that were shown to be successful for the management of these infections are very limited; cidofovir being the most commonly recommended drug. Topical [32–34], intravenous [35, 36], or intralesional [37] cidofovir administrations have been successfully used in the treatment of ACVr and/or PFAr infections. Furthermore, cidofovir therapy provided significant benefits in lesion healing, virological effect, and pain reduction in a randomized, double-blind, placebo-controlled trial of cidofovir gel for the

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Figure 5. Diagram of the herpes simplex virus type 1 (HSV-1) DNA pol. Locations of functional sites on the HSV-1 DNA pol and linear N terminus to C terminus configuration of the polypeptide are shown. Solid boxes show regions numbered I–VII based on conservation among the DNA pols and δ-region C, a region showing slight conservation. An exhaustive review of the literature was done to illustrate the amino acid changes previously described that are known to be associated with genetic polymorphisms (green) or drug-resistance (red). Mutations that confer drug-resistance located in the conserved regions are shown in dark grey boxes. Mutations found in other regions of the HSV-1 DNA pol are shown in white boxes. Mutations found in the present study are underlined and shown in black.
treatment of ACV-unresponsive mucocutaneous HSV infection in AIDS patients [38]. The use of 3% cidofovir in a saline rinse for ACV and PFA refractory mucosal HSV infection in a severely immunosuppressed patient was recently reported [39]. This route of cidofovir delivery could have been used in our patient; however, because she developed severe leukemia-related complications, antiviral treatment was stopped.

Other therapies have been utilized in treating ACV-resistant HSV infections. Imiquimod, a toll-like receptor agonist that enhances the innate immunological responses, has shown efficacy in the treatment of recalcitrant HSV infections when administered topically [40]. An orally bioavailable prodrug of cidofovir (CMX-001) that lacks nephrotoxicity may be an alternative therapy for resistant HSV infections [41]. The 5-azacytosine derivative of cidofovir, with a spectrum of activity comparable to cidofovir, represents another therapeutic option [42]. Novel antiviral agents with a different mode of action (such as helicase–primase inhibitors [43, 44]) are potential alternatives for the treatment of multidrug-resistant HSV infections.

Herpesvirus infections are important causes of morbidity and mortality after allogeneic HSCT because T-cell–mediated immune responses are the main factors for controlling herpesvirus infections and these responses are more severely suppressed after allogeneic transplantation. The role played by the innate immune system has recently been exemplified in humans [45]. The critical involvement of adaptive T-cell immunity in neurological complications of HSV was also shown in mice [46]. The results of our case study are in line with these discoveries and demonstrate that virus monitoring in patients under immunosuppressive therapy is advised. Prophylaxis with ACV is indicated in all HSV-seropositive HSCT recipients and in some autologous patients presenting high risk for mucositis [47]. Prophylaxis should be continued throughout the aplastic phase and needs to be adjusted for each patient. HSV recurrences frequently occur soon after prophylaxis is stopped and require therapy. Acyclovir resistance is not an infrequent problem in the HSCT population and is often seen after prolonged ACV prophylaxis or treatment in patients with impaired immunity. A prevalence of ACV resistance ranging from 4.1% to 10.9% and up to 36% has been reported in this group of patients [20, 21, 48, 49].

Duan et al showed, in the cornea (considered as an immune-privileged site), an unexpected diversity and unpredictable temporal pattern of the ACV susceptibility profile of sequential corneal isolates from patients with recurrent herpetic keratitis [50]. Thus, in immune-privileged sites and in severely immunosuppressed patients, HSV can generate different viral populations. This viral evolution may lead to the appearance of several viral populations that can coexist in the patient, leading to multiple drug resistances.

In conclusion, our data suggest that multiple temporal virus sampling is required to evaluate the emergence of viral drug resistance. Understanding the heterogeneity within virus populations found in a lesion, that is, coexistence of several drug-resistant viruses in the presence or absence of wild-type virus, is pivotal because it will determine the clinical response to antiviral treatment.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.
Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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