**Original Article**

**Long-Term Observation of Herpes Simplex Virus Type 1 (HSV-1) Infection in a Child with Wiskott-Aldrich Syndrome and a Possible Reactivation Mechanism for Thymidine Kinase-Negative HSV-1 in Humans**

Tomoyuki Shiota, Ichiro Kurane, Shigeru Morikawa, and Masayuki Saijo*

*Department of Virology I, National Institute of Infectious Diseases, Tokyo 162-8640, Japan*

(Received September 21, 2010. Accepted January 24, 2011)

**SUMMARY:** Herpes simplex virus type 1 (HSV-1) infections in a child with congenital immunodeficiency syndrome were observed over a 10-year period. The child suffered from recurrent and severe HSV-1 mucocutaneous infections. He frequently suffered from acyclovir (ACV)-resistant (ACVr) HSV-1 infection in the later phase of his life, especially after the episode of ACV-sensitive (ACVs) HSV-1 infection. Virological analyses on the HSV-1 isolates recovered from this patient revealed that all the ACVs HSV-1 isolates were thymidine kinase (TK)-negative (TK-) due to a single cytosine (C) deletion within the 4-C residues (positions 1061 to 1064) in the TK gene, indicating that the recurrent TK-/ACVs HSV-1 infections throughout the patient’s life were due to the identical ACVs HSV-1 strain. Furthermore, it was found that the ACV-sensitive (ACVs) isolate recovered from the skin lesions that appeared between the episodes of ACVr infection at the ages of 8 and 9 contained ACVr HSV-1 with the same mutation in the TK gene. These results indicate that, although TK activity is required for reactivation of TK+/ACVs HSV-1 from latency and TK+/ACVr HSV-1 is unable to reactivate from latency, the TK-/ACVr HSV-1 strain isolated herein reactivated in this patient, possibly by using the TK activity induced by the latently co-infected TK-/ACVr HSV-1.

**INTRODUCTION**

Herpesviruses are common causative agents for life-threatening infections in patients with immunodeficiency, the majority of whom have already been latently infected with herpesvirus. Indeed, reactivation occurs frequently in these patients. Acyclovir (ACV), which is phosphorylated by virus-induced thymidine kinase (TK) to form ACV monophosphate (ACVMP), and subsequently to ACV diphosphate (ACVDP) and ACV triphosphate (ACVTP) by cellular enzymes, has been widely used to treat such patients. ACVTP is incorporated into viral DNA, resulting in termination of viral DNA chain elongation and inhibition of virus replication (1). ACV-resistant (ACVr) herpesviruses have a mutation in the TK and/or DNA polymerase gene, although the former is far more frequent.

We reported previously that the ACV-sensitive HSV virus type 1 (HSV-1) recovered from a patient with a congenital immunodeficiency (Wiskott-Aldrich syndrome [WAS]) was viral TK-negative (TK-) and that this TK-/ACV-sensitive HSV-1 strain could reactivate in humans (2). Likewise, it has been reported that although TK- HSV-1 can establish latency in mouse trigeminal ganglia, it could not be reactivated in this mouse animal model (3,4). The mechanism of reactivation for TK+/ACV-sensitive HSV-1 from latency in humans remains unclear.

The life-long ACV-sensitive (ACVs) and/or ACVr HSV-1 infections in our WAS patient were observed clinically and virologically. Indeed, numerous ACVs or ACVr HSV-1 isolates were recovered at different stages during the approximately 10-year period between primary infection and death. In the present study, the entire course of these HSV-1 infections is presented. Furthermore, the existence of TK-/ACV-sensitive (ACVs) HSV-1 with an identical mutation to those demonstrated in the sequential TK+/ACVr HSV-1 isolates from this patient was proven by using specific and unique strategies to elucidate the reactivation mechanism of TK-/ACV-sensitive HSV-1 from latency in humans.

**MATERIALS AND METHODS**

**Patient:** The patient was a boy with WAS, an X-linked recessive disorder characterized by immunodeficiency, thrombocytopenia, and eczematoid dermatitis. He was infected primarily with HSV-1 at the age of 3, and thereafter suffered from frequent recurrences of ACV-sensitive (ACVs) and ACVr HSV-1 infections throughout his life. He experienced an episode of ACVr HSV-1 infection at the ages of 8 and 9. Specific episodes of HSV-1 infections during the patient reported previously (2,5,7-9).

**Compounds:** ACV and foscarnet (Sigma Chemical Co., St. Louis, Mo., USA) were used.

**Cells and viruses:** Human embryonic lung fibroblast cells and green monkey kidney cell line cells (Vero cells) were used for virus isolation. Virus isolation and identification were performed as described previously (2). The HSV-1 isolates recovered from the patient were used in this study after passaging to P1 or P2 (Table 1).

**Plaque reduction assay:** The susceptibility of HSV-1 isolates to ACV and foscarnet was assessed by plaque reduction assay in Vero cells, as described previously (10).

**Generation and selection of ACV-sensitive HSV-1 clones:** ACV-sensitive HSV-1 isolates were obtained by propagating HSV-1 in the presence of ACV. ACV-sensitive HSV-1 isolates were further propagated in Vero cells, and ACV-resistant (ACVr) clones were selected by the plaque reduction assay in the presence of ACV (10).
HSV-1 clones were generated from TA1, TAS, TA4, and 100-fold diluted TA4 isolate solutions (Table 1, Fig. 1A). The concentration of ACV in the culture medium was increased in a stepwise manner from 0.1 to a final concentration of 3.0 µg/ml (0.1, 0.3, 1.0, and 3.0 µg/ml). The infectious dose in the original virus solutions of the TA1, TAS, and TA4 isolates was confirmed to be approximately 2 × 10^7 plaque-forming units (PFU)/ml. A 0.1-ml aliquot of the virus solution was inoculated in the first step for selection, indicating that selection started from an infectious dose of approximately 2 × 10^6 PFU. After the final selection step, 19–26 clones were obtained by 3-fold plaque-purification from each of these isolate solutions (see Table 2). The method used previously to develop ACV^r clones (11) is shown in Fig. 1B.

Nucleotide sequencing: The nucleotide sequence of the TK gene was determined for the HSV-1 isolates and the generated ACV^r HSV-1 clones by cycle sequencing of the PCR-generated products amplified from the purified DNA (2).

RESULTS

Entire clinical course of HSV-1 infections: The entire course of the HSV-1 infections, including HSV-1 isolation, anti-viral treatment, and specific BMT treatment, presented by our patient is shown schematically in Fig. 2. The patient suffered from a primary HSV-1 infection in the form of gingivostomatitis at the age of 3, at which time the TA1 isolate was recovered. Thereafter, the patient suffered from recurrent episodes of herpes simplex, which were treated with oral or intravenous administration of ACV. A severe mucocutaneous HSV-1 infection, which was treated by continuous administration of ACV (2 mg/kg/h), occurred around his left eye at the age of 7 (Age-7 ACV^s infection) (7). The TAS isolate was recovered during the course of this therapy and subsequently confirmed to be ACV^s (2,7,8). Despite prophylactic treatment with oral ACV (80 mg/kg/day, divided into 4 doses) as a result of this episode, the patient subsequently suffered a severe recurrence of herpes simplex that presented with lesions on his face, arms, genitalia, back, and feet at age 8 (Age-8 ACV^r infection). Although ACV was administered intravenously and continuously, the skin lesions did not respond. A TAR isolate was recovered and found to be ACV^r (2,7,8). Despite prophylactic treatment with oral ACV (80 mg/kg/day, divided into 4 doses) as a result of this episode, the patient subsequently suffered a severe recurrence of herpes simplex that presented with lesions on his face, arms, genitalia, back, and feet at age 8 (Age-8 ACV^r infection). Although ACV was administered intravenously and continuously, the skin lesions did not respond. A TAR isolate was recovered and found to be ACV^r (2,7,8). Subsequently, the patient was treated with vidarabine (15–20 mg/kg/day, intravenously), which resulted in complete resolution. The prophylactic use of oral ACV was terminated. The herpes simplex again relapsed and a TA4 isolate was recovered from vesicular lesions on his face 6 months after complete resolution of the Age-8 ACV^r infection. The relapsed herpes simplex was treated with oral administration of ACV as the TA4 isolate was demonstrated to be ACV^r. The patient again suffered from intractable, ulcerative, and proliferative herpes simplex around the left eye at age 9 (Age-9 ACV^r infection) (2,7). This infection was treated by continuous administra-

Table 1. HSV-1 isolates recovered from the patient and their characteristics

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Age of patient at isolation</th>
<th>Site of isolation</th>
<th>Sensitivity to ACV</th>
<th>Sensitivity to Foscarnet</th>
<th>Mutation in the TK gene</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA1</td>
<td>3</td>
<td>Oral cavity</td>
<td>S</td>
<td>S</td>
<td>None^2</td>
<td>Primary infection</td>
</tr>
<tr>
<td>TAS</td>
<td>7</td>
<td>Left orbital lesion</td>
<td>S</td>
<td>S</td>
<td>None</td>
<td>Reactivation</td>
</tr>
<tr>
<td>TAR</td>
<td>8</td>
<td>Left orbital lesion</td>
<td>R</td>
<td>S</td>
<td>TAR-type^3</td>
<td>Reactivation</td>
</tr>
<tr>
<td>TA4</td>
<td>8.5</td>
<td>Left orbital lesion</td>
<td>S</td>
<td>S</td>
<td>None</td>
<td>Reactivation</td>
</tr>
<tr>
<td>TA5</td>
<td>9</td>
<td>Left orbital lesion</td>
<td>R</td>
<td>S</td>
<td>TAR-type^3</td>
<td>Reactivation</td>
</tr>
<tr>
<td>R98/0</td>
<td>13</td>
<td>Facial and genital lesions</td>
<td>R</td>
<td>S</td>
<td>TAR-type^3</td>
<td>Reactivation</td>
</tr>
</tbody>
</table>

1): The nucleotide sequence of the isolate was determined as described in the Materials and Methods section from the purified DNA from the isolate solution without any plaque purification step.

2): The nucleotide sequence is deposited in GenBank under the accession number of AB047358.

3): The nucleotide sequence is deposited in GenBank under the accession number of AB047365.
tion of ACV, but the lesions did not improve. Isolate TA5 was recovered during the ACV treatment and was demonstrated to be ACV-resistant. Combined therapy with continuous administrations of ACV (2 mg/kg/h) and vidarabine (15 mg/kg/day), which resulted in a marked improvement, was initiated (Fig. 2). The incidence of severe mucocutaneous skin infections due to ACV-resistant HSV-1 increased significantly after the Age-9 ACV-resistant infection. At the age of 13, an allogeneic BMT from an HLA-matched unrelated donor resulted in severe and generalized HSV-1 skin infections due to ACV-resistant HSV-1 (5). HSV-1 R98/0 was isolated from the lesion on day 33, taking the day on which transplantation was performed as day 0, and subsequently confirmed to be resistant to both ACV, although it was sensitive to foscarnet. Foscarnet treatment was therefore initiated. The lesions responded well to the treatment, but did not resolve completely. Foscarnet-resistant HSV-1 emerged in the later phase of this infection (5). The patient died of PML caused by JC virus (6).

Sensitivity to ACV and nucleotide sequence of the TK gene from HSV-1 isolates: The TA1, TAS, and TA4 isolates were confirmed to be ACV-sensitive, whereas other isolates (TAR, TA5, and R98/0) were confirmed to be ACV-resistant. The TK gene in these isolates had the same mutation, namely a frameshift mutation due to a single cytosine (C) deletion within the 4-C residues at nucleotide positions 1061 to 1064. This type of mutation is defined as a TAR-type mutation.

Selection of ACV-resistant clones from the TA1, TAS, and TA4 isolates: A total of 26, 19, 22, and 20 clones, respectively, were plaque-purified from the virus solutions selected by growing the TA1, TAS, TA4, and a 100-fold diluted TA4 isolates in Vero cells cultured in MEM-2FBS with ACV (Fig. 1A). All the clones generated were confirmed to be ACV-resistant.

Determination of the nucleotide sequences of the TK gene for the ACV-resistant clones derived from each isolate: The mutation patterns detected in the nucleotide sequence of the TK genes of the TA1- and TA4-derived ACV-resistant clones are summarized in Table 2. The mutation pattern detected in the nucleotide sequence of the TK gene from the plaque-purified ACV-resistant clones studied in the previous report (11) is also included for comparison (Table 2). Only 2 of the 24 clones (8.3%) derived from the plaque-purified TAS studied in the previous report (11) had the TAR-type mutation (Table 2). The TAR-type mutation was identified in 1 of 26 clones derived from the TA1 isolate. The percentages of ACV-resistant clones with a nucleotide deletion/insertion or a nucleotide substitution among the TA1-derived clones (42 and 58%, respectively) were approximately similar to those from the plaque-purified TA4-derived clones (50 and 50%, respectively).

Table 2. Mutation patterns in the nucleotide sequence of the TK genes of ACV-resistant clones generated from plaque-purified TAS, TA1, TAS, TA4, and 100-fold-diluted TA4 isolate solutions

<table>
<thead>
<tr>
<th>Mutation pattern</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA1</td>
</tr>
<tr>
<td>C insertion within 4-C residues (1061–1064)</td>
<td>1</td>
</tr>
<tr>
<td>G insertion within 7-G residues (430–436)</td>
<td>7</td>
</tr>
<tr>
<td>G deletion within 7-G residues (430–436)</td>
<td>1</td>
</tr>
<tr>
<td>C insertion within 6-C residues (548–553)</td>
<td>1</td>
</tr>
<tr>
<td>C deletion within 6-C residues (548–553)</td>
<td>1</td>
</tr>
<tr>
<td>Nucleotide insertion/deletion of other positions</td>
<td>0</td>
</tr>
<tr>
<td>Early appearance of stop codon due to nucleotide substitution</td>
<td>0</td>
</tr>
<tr>
<td>Single amino acid substitution due to nucleotide substitution</td>
<td>15</td>
</tr>
<tr>
<td>No mutation</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
</tr>
</tbody>
</table>

The mutation patterns determined in our previous study are also shown (11).

1) Nucleotide positions counted from the initiation codon.

2) Insertion or deletion of a single nucleotide was demonstrated in other regions of the nucleotide homopolymer stretch.

3) The clones resistant to ACV with no mutations in the TK gene were speculated to have amino acid substitutions in the DNA polymerase gene as the result of nucleotide substitutions (19).
tively). On the other hand, the TAR-type mutation was identified in 15 of 19 TAS-derived clones (79%) and in 6 of 23 TA4-derived clones (26%). Interestingly, this mutation, a single C-insertion within the 6-C residues (position 548-553), was demonstrated in 6 of the 22 TA4-derived clones (26%), whereas the same mutation was demonstrated in only 0% (0 of 24) and 3.8% (1 of 26) of the plaque-purified TAS- and TA1-derived clones, respectively. To confirm whether ACVr clones with the TAR-type mutation were obtained by the selection of preexisting mutants or by the generation of mutants from ACV HSV-1 strains during culture, a 100-fold diluted TA4 isolate solution was treated in the same manner as the original TA4 isolate solution. The TAR-type mutation and the single C-deletion within the 6-C residues (position 548-553) were demonstrated in 4 (25%) and 3 (15%) of 20 clones, respectively, thus indicating that the ACVr clones with these mutations preexisted in the TA4 isolate solution.

**DISCUSSION**

One of the purposes of this study was to illustrate the entire clinical course of severe HSV-1 infections in a child with a congenital immunodeficiency. To the best of our knowledge, this kind of study is relatively rare. In the later phase of his life, especially after the Age-9 ACVr infection, the incidence of ACVr HSV-1 infections increased significantly, and severe and intractable skin infections caused by ACVr HSV-1 continued to recur frequently until his death. This study indicates that the treatment of herpesvirus infections in patients with immunodeficiency is complicated, difficult, and never-ending. Further studies on the development of therapeutic strategies for such infections, such as the introduction of newly developed helicase primase inhibitors or a combination therapy with a nucleoside analogue such as ACV and the newly developed helicase primase inhibitors are therefore necessary (12-14).

Another purpose of this study was to elucidate the reactivation mechanism of TK+/ACVr HSV-1 in humans. Several previous reports, including ours, have shown that TK+/ACVr HSV-1 can reactivate in humans (2,15,16). Indeed, evidence for the reactivation of TK+/ACVr HSV-1 in humans was confirmed by the existence of the same mutation in the nucleotide sequence of the TK gene in the initial TK+/ACV HSV-1 isolate and the subsequent TK+/ACV HSV-1 isolate. However, the mechanism underlying TK+/ACV HSV-1 reactivation remains uncertain.

All the HSV-1 ACVr isolates had the identical nucleotide sequence in the TK gene. Likewise, all the ACVr HSV-1 isolates also had an identical nucleotide sequence. As only one nucleotide deletion was demonstrated in the TK gene of ACVr HSV-1 isolates compared with ACV HSV-1 recovered in this patient, the ACVr HSV-1 isolates are likely to have originated from the ACV HSV-1 isolates. Furthermore, the nucleotide sequence in the DNA polymerase gene of each isolate was identical, except for that of isolate R98/3, which was isolated during the course of the BMT process and was found to be resistant to foscarnet (5). We also found that the nucleotide sequence in the TK gene of ACVr HSV-1 isolate was identical to that in the TAS recovered from this patient in only 1 of 10 HSV-1 strains (9 clinical isolates and a laboratory KOS strain) (2), thereby suggesting that all the episodes of HSV-1 infection in this patient, except for the primary infection at the age of 3, were due to reactivation of the same virus from latency.

The ACVr TA4 isolate was recovered 6 months after complete resolution of the Age-8 ACVr infection. The ratios of ACVr clones with TAR-type mutation to the total number of TA4-derived ACVr clones tested and to that of the 100-fold diluted TA4-derived ACVr clones tested were significantly higher than that of the plaque-purified TAS-derived ACVr clones. These results indicate that ACVr HSV-1 with the TAR-type mutation preexisted in the TA4 isolate solution and suggest that the mutant reactivated simultaneously upon reactivation of TK+/ACVr HSV-1.

TK+/ACVr HSV-1 was previously found to establish latency, but not reactivate, in a murine model (3,4). Furthermore, viral TK activity was found to be required for reactivation of TK+ HSV-1 from latency in the sensory nerve ganglia (17). Likewise, the TK protein expressed in Vero cells infected with the plaque-purified HSV-1 with the TAR-type mutation was found to be insoluble, have an aggregative form, and did not induce any TK activity (11). The 50% lethal dose (LD50) of the purified HSV-1 TAR, as determined by intracranial inoculation of the virus into mice, which is an indicator of the expression of TK activity, was >40,000 PFU, whereas that of the purified TAS was 0.7 PFU (8). Furthermore, it was confirmed that the TK+/ACVr HSV-1 with the TAR-type mutation in the TK gene failed to reactivate from latency in a murine model (8). TK+/ACVr HSV-1 with the TAR-type mutation should have little or no ability to reactivate from latency, although in this patient it was found to do so. A possible reactivation mechanism for this patient is as follows. Individual neuronal cells in this patient may have been latently co-infected with both TK+ ACVr HSV-1 and TK+/ACVr HSV-1, as reported previously in another patient (16). The TK activity induced by TK+/ACVr HSV-1 when it reactivated could then have compensated for the lack of TK activity that had prevented the TK+/ACVr HSV-1 from reactivating from latency, thereby allowing it to reactivate. A similar phenomenon was demonstrated in a murine model (18).

To confirm this mechanism, the mutation patterns in the TK gene in the HSV-1 solution, which does not contain any ACVr HSV-1 with specific mutations in the TK gene, would need to be studied using independent ACVr HSV-1 solutions, which fortunately can be prepared by plaque-purification from ACVr HSV-1. The mutation patterns in the TK gene in the plaque-purified TAS-derived ACVr clones obtained in the previous report were therefore included in this study as control (11). The mutation patterns in the TK gene in the TA1-derived ACVr clones were found to be almost identical to those of the plaque-purified TAS-derived ACVr clones, thus indicating that ACVr HSV-1 was not present in the virus population of the TA1 isolate and that the viruses that replicated in the lesions during primary infection did not contain any ACVr clones. The ACVr HSV-1 with the TAR-type mutation was confirmed to be present for the first time when the patient experienced the Age-7 ACVr infection. There are two possibilities regarding the timing of emergence of the ACVr HSV-1 strain: the TK+/ACVr HSV-1 emerged and became established latency during the course of repeated ACV treatment prior to the Age-7 ACVr infection, or TK+/ACVr HSV-1 emerged during the course of ACV treatment for the Age-7 ACVr infection.

A possible mechanism to explain the frequent recurrence of TK+/ACVr HSV-1 infections in the later phase of the patient’s life is shown schematically in Fig. 3. The frequent
recurrence of ACVr HSV-1 infections allowed the TK+/ACVs HSV-1 to latently infect the sensory nerve cells in the ganglia, thereby increasing the ratio, and absolute number, of latently infected TK+/ACVs HSV-1 in sensory nerve cells in the sensory nerve ganglia. This phenomenon might contribute to the increased incidence of TK+/ACVs HSV-1 infections.

Another ACVr HSV-1 strain with a single C-insertion within the 6-C residues (position 548–553) was detected in the TA4 and 100-fold diluted TA4 isolate solutions, thus suggesting that the ACVr HSV-1 strain with this mutation emerged and established latency.

In summary, HSV-1 infections in a child with WAS were observed clinically and virologically for approximately 10 years from the primary infection to his death. All the ACVr HSV-1 strains recovered throughout the patient’s life had the same mutation in the TK gene. All the isolates that were subsequently determined to be ACVr contained ACVr HSV-1 with the same mutation in the TK gene. TK+/ACVs HSV-1, which itself lacks the ability to reactivate from latency, might reactivate using the TK activity induced by the reactivation from latency of TK+/ACVs HSV-1 with which the patient was co-infected.

Acknowledgments We would like to thank Ms M. Ogata, Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan, for

![Diagram](image-url)
her technical assistance in the study.

This study was supported by a Grant-in-Aid for Scientific Research (10770378 and 12770416) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant-in-Aid from Research on Measures for Emerging and Reemerging Infections (Intractable Infectious Diseases in Organ Transplant Recipients [H21-Shinko-Ippan-009]) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest None to declare.

REFERENCES


