Biological and Biochemical Characterization of Clinical Isolates of Herpes Simplex Virus Type 2 Resistant to Acyclovir

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A series of clinical isolates of herpes simplex virus type 2 were taken from a patient with chronic lymphocytic leukemia. Acyclovir (ACV) susceptibility assays revealed that some isolates were resistant to ACV and cross-resistant to ganciclovir but not to phosphonoacetic acid. The nature of the resistance was examined further. A number of cloned variants were generated, and thymidine kinase and DNA polymerase assays were carried out. Variants that were resistant to ACV were found to be thymidine kinase deficient. Evidence for alteration in the DNA polymerase was not found when ACV triphosphate or phosphonoacetic acid was used as the inhibitor. In vivo studies with the plaque-purified viruses showed that ACV resistance was associated with a reduced neurovirulence. In a zosteriform model, virus resistant to ACV was unable to induce secondary spread in the same dermato-me, to invade the peripheral nervous system or the central nervous system, or to establish latent infections.

The efficacy and selectivity of acyclovir (ACV) has resulted in its becoming the drug of choice for the treatment of herpes simplex virus (HSV) and varicella-zoster virus infections (13). Nevertheless, concern has been expressed about the potential for resistant virus to appear in ACV-treated individuals. Laboratory investigations with HSV have revealed that resistance to ACV may develop under specific selection pressure. Characterization of laboratory-generated resistant mutants indicates that resistance arises as a result of loss or alteration of thymidine kinase (TK) activity or alteration in the virus DNA polymerase (4, 15, 18, 23). Virus isolates recovered from clinical trials of ACV in immunocompetent patients have been monitored and have shown little evidence of alteration in their susceptibility to ACV (2, 8). However, there have been a small number of reports of the isolation of virus with reduced susceptibility, particularly from bone marrow transplant patients and leukemic patients undergoing induction chemotherapy (2, 3, 8, 9, 16, 24, 30).

HSV clinical isolates with reduced susceptibility to ACV have resulted from a deficiency or alteration in virus TK (5), with one recent exception, when virus with an altered DNA polymerase was identified (7, 22). There is no clear indication of the significance of the isolation of resistant virus or its relationship with the clinical course of a disease (12, 20), their is often being no correlation between the clinical response to ACV and the isolation of resistant virus (2, 11). Virus recovered during recurrences subsequent to an episode from which resistant virus was isolated have all retained a phenotype reflecting the susceptibility of the original infecting virus which initiated latency (1, 25, 27). Thus, such recurrences would be expected to be amenable to ACV therapy. Evidence in animal models with mutants displaying a TK-deficient phenotype suggest that the resistant clinical isolates recovered would not establish latent infections and would thus be unlikely to be transmitted (10, 14, 29).

Few reports detailing the susceptibility of sequential isolates from one patient have been made (21, 24). During routine susceptibility monitoring, isolates from a 75-year-old male with chronic lymphocytic leukemia who received multiple courses of ACV therapy for persistent perianal herpes were characterized. These isolates exhibited an unusual profile of susceptibility to ACV. Two early isolates were susceptible to ACV and ganciclovir (9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine), whereas subsequent isolates taken during prolonged exposure to ACV were less susceptible. The aim of the work described in this report was to determine the susceptibility of all the isolates received and relate this to their biochemical properties, their pathogenicity, and the ability of the isolates to cause latent infections in animal models.

MATERIALS AND METHODS

Patient details. The patient was a 75-year-old male with chronic lymphocytic leukemia, who developed a severe perianal herpesvirus infection in October 1982. This was successfully treated with intravenous (i.v.) ACV at 1.5 g per day, given for 5 days. The infection recurred in February 1983 and was again successfully treated with i.v. ACV. Over the next 6 months, the lesions gradually recurred and oral ACV at 1 g per day was given, but no clinical improvement was observed and virus was isolated (Fig. 1). Treatment was increased to i.v. ACV at 2.25 g per day, but was again unsuccessful. A course of topical phosphonoformic acid was given, but the patient remained culture positive. By January 1984, the lesions had extended to the left thigh and i.v. ACV at 3 g per day was given, which ameliorated the lesions. In February 1984, cecal carcinoma was diagnosed and a hemicolectomy was performed. A relapse of the herpesvirus infection in May 1984 was treated with i.v. ACV at 2.25 g per day, with limited success. Finally, oral ACV at 2.4 g per day was prescribed, but the patient remained culture positive. Further virus isolates were unobtainable. In September 1984 he received courses of Leukeran (Burroughs Wellcome Co., Research Triangle Park, N.C.) and prednisone for his leukemia, but he died in October 1984.

Virus isolates. Virus isolates were cultured in human embryonic fibroblasts and received as frozen cell culture extracts in our laboratory. They were grown in Vero cells.
(Flow Laboratories, Irvine, Scotland), and the resultant crude virus extracts were then aliquoted and stored at −70°C. Susceptibility assays were performed on pass 2 or 3 material only. The type 2 strains BRY, BRY TK, 186R (a phosphonoacetic acid [PAA]-resistant type 2 herpesvirus), and its parent strain 186 were used as controls.

**Compounds for antiviral assays.** ACV and ganciclovir were prepared by the Wellcome Chemical Works, Dartford, England, and phosphonoacetic acid (PAA) was supplied by P. Furman (Burroughs Wellcome Co.). All compounds were prepared as 1 or 10 mM (PAA) solutions in deionized water, filter sterilized, aliquoted, and stored at −20°C.

**Viral susceptibility assays.** A standard plaque reduction assay was used to determine the susceptibility of the isolates (6, 27). Preformed Vero cell monolayers in Falcon 24 multiwell plates (Becton Dickinson, Oxford, England) were infected with 100 to 150 PFU per well. After a 1-h adsorption at 37°C in a 5% CO₂ atmosphere, the wells were overlaid with serial twofold dilutions of the test compound in 1% carboxymethyl cellulose containing 10% Glasgow modified Eagle medium (Northumbria Biologicals, Cramlington, England), 0.11% sodium bicarbonate, 2 mM L-glutamine (GIBCO), and 100 U each of penicillin and streptomycin (GIBCO) per ml. After a 72-h incubation, the cells were fixed and stained with 0.5% methyl violet. Plaque counts were expressed as a percentage of the virus control and used to construct dose-response lines from which the 50% inhibitory concentrations were determined from the resultant linear regression lines.

**Production of cloned variants.** Serial 0.5-log dilutions of each isolate were used to infect 96-well plates (Falcon) seeded with Vero cells. The cells were overlaid and incubated as before. Cultures were observed closely for plaque formation; wells containing single plaques were marked. After a further 24 to 48 h, any secondary plaque formation was noted and single plaques were picked. Infected cells were sonicated, and an aliquot was added to Vero cell monolayers. Crude virus infected stocks were prepared as for the parent isolates, and sensitivity assays were carried out. Two clones from each isolate were selected for further analysis.

**TK assay.** Crude enzyme extracts were prepared in TK-negative BHK (BuBHK) cells. The measurement of the nucleoside-phosphorylating activity was based on published methodology (17). The extract was prepared following an 18-h incubation of cells infected with 10 PFU per cell. Infected cells were disrupted in 0.01 M Tris buffer (pH 7.4) and sonicated, and aliquots were assayed at 37°C in 0.02 M phosphate buffer with 5 mM magnesium chloride, 5 mM ATP, and 30 μM [14C]thymidine (Amersham International plc, Amersham, England) as substrate. The conversion of thymidine to phosphorylated derivatives was expressed as picomoles of phosphorylated product per 10⁵ or 10⁶ cells.

**DNA polymerase assay.** A crude enzyme extract was prepared in BuBHK cells, as for the TK assay, infected cells being suspended in 250 mM phosphate buffer (pH 7.5) with 1 mM EDTA, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (Sigma, Poole, England). The reaction mix contained 50 mM Tris buffer (pH 7.5), 200 mM KCl, 4 mM MgCl₂, 0.5 mM dithiothreitol, 0.0125 mg of activated calf thymus DNA (Lorne Diagnostics Ltd., Bury St. Edmunds, England), and 2 mM TTP, dATP, and dCTP, with 5 μM [8-β-H]dGTP at 50 μCi/ml (Amersham), plus 5 μl of enzyme. The total volume of 100 μl contained ACV triphosphate (ACV-TP) or PAA as inhibitor. ACV-TP was used at 0, 0.05, 0.5, 3.0, and 10.0 μM. After incubation for 5 min at 37°C, 50-μl aliquots were spotted onto DE81 disks (Whatman, Maidstone, England), which were washed three times with 0.3 M ammonium formate made pH 7.8 with ammonia. Radioactivity was determined by scintillation counting with Optiscint Safe scintillant (LKB, Croydon, England).

**Restriction enzyme analysis.** DNA from selected clones was prepared by infecting 10⁵ BHK21 cells with 0.1 PFU per cell. After a 48-h incubation, cells were lysed in 10 mM Tris (pH 8.0) with 10 mM EDTA and 0.25% Triton X-100 (Sigma). Following gentle mixing, 0.2 M NaCl was added, and nuclei were pelleted at 450 × g. The supernatant was treated with 0.2% sodium dodecyl sulfate and 1 mg of pronase per ml and incubated at 37°C for 4 to 6 h. The DNA was extracted twice with phenol-chloroform and once with chloroform and then precipitated with 2.5 volumes of ethanol at −20°C. After the DNA had been pelleted, it was dried, dissolved in 400 μl of water, and mixed with 400 μl of 5 M LiCl to precipitate RNA. After centrifugation, the supernatant was mixed with ethanol at −70°C. The DNA was spun down and dissolved in TE (10 mM Tris, 1 mM EDTA), with 0.2 M NaCl added. After the nucleic acid had been precipitated with 2.5 volumes of cold ethanol, the DNA was spooled and dissolved in 200 μl of TE. The DNA was digested with a number of restriction enzymes (GIBCO/Bethesda Research Laboratories) and analyzed on 0.8% agarose gels (Miles Laboratories, Slough, England).

**In vivo studies.** Studies of neurovirulence were performed with groups of 10 CD1 mice weighing 15 to 18 g (Charles River Breeding Laboratories, Manston, Kent, England). The

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**FIG. 1.** Patient details.
mice were inoculated with serial 10-fold dilutions of virus by the intracerebral route, and survival times were recorded. Pathogenicity was further studied by using a zosteriform (26) model of HSV in imbed BALB/c mice (Animal Health Control, Wellcome Research Laboratories, Beckenham, England). A primary infection was induced on the depilated upper flank by scarification through 10 μl of virus suspension containing 10⁶PFU. The development of secondary zosteriform spread of infection and subsequent survival times were recorded.

RESULTS

Viral susceptibility assays. (i) Isolates. The isolates were identified as being HSV-2, and this was confirmed subsequently by restriction enzyme analysis. Results from at least two separate plaque reduction assays against ACV, ganciclovir, and PAA are summarized in Table 1. Isolates 1 and 2, taken before the courses of ACV therapy, were susceptible to ACV, ganciclovir, and PAA, although isolate 2 was two- to threefold less susceptible to ACV than isolate 1 was. These values, however, fall within the ranges seen for susceptible HSV-2 isolates in assays in Vero cells (5). Isolates 3, 4, and 5, taken during the protracted period of HSV infection, were resistant to ACV and ganciclovir. During a period when ACV therapy was stopped, a further isolate (no. 6) was obtained, which was of increased susceptibility to ACV and ganciclovir and was also susceptible to PAA. The final isolate, isolate 7, taken before a successful course of i.v. ACV treatment, was susceptible to all compounds tested.

(ii) Plaque-purified clones. Plaque-purified clones from each isolate were produced, and their susceptibilities to ACV were determined. The number of resistant clones found compared with the total number produced for each isolate is summarized in Table 2. Isolates 1 and 6 were clearly mixtures of ACV-susceptible and ACV-resistant viruses, whereas all the clones from isolates 3, 4, and 5 were resistant to ACV. For isolates 2 and 7, all the clones were susceptible to ACV. Two clones from each isolate were selected and further characterized. The susceptibility data (Table 3) give the average 50% inhibitory concentrations for at least two separate assays. For isolates 1 and 6, a susceptible and a resistant clone were evaluated. Clones resistant to ACV were correspondingly resistant to ganciclovir, although for 4A and 5B, resistance to ganciclovir was four times that of resistance to ACV. Despite some variability, the data for PAA showed that all the clones tested were susceptible to this compound.

TABLE 2. ACV susceptibilities of the cloned variants of the parent isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>No. of clones</th>
<th>No. resistant to ACV&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>5</td>
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<tr>
<td>3</td>
<td>15</td>
<td>5</td>
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<td>4</td>
<td>5</td>
<td>7</td>
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<td>5</td>
<td>7</td>
<td>7</td>
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<tr>
<td>6</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Resistance is defined as 50% inhibitory concentrations greater than 10 μM.

TK assay. The TK phosphorylating activity of the seven isolates is given in Table 3. For the three ACV-resistant isolates, isolates 3, 4, and 5, no activity was detected. With the three susceptible isolates, isolates 1, 2, and 7, activity comparable to that of wild-type controls was seen. Isolate 6, of reduced susceptibility, showed only 20% TK activity compared with the ACV-susceptible isolates (data not shown). Clones from this isolate were of mixed susceptibility to ACV and ganciclovir, whereas for the susceptible variants, wild-type TK levels were expressed. The TK-negative control BRY TK<sup>−</sup> was included and showed activity equivalent to that of the mock-infected extract. Mock-infected and TK-negative extracts showed somewhat between 0 and 5% of the wild-type activity and thus represent the limits of detection of the assay.

DNA polymerase assay. The susceptibilities of the DNA polymerase preparations, with either ACV-TP or PAA as inhibitor and expressed as 50% inhibitory concentrations, are summarized in Table 4. The HSV-2 virus 186R and its PAA-resistant variant 186R are included as controls. A 10-fold decrease in the susceptibility of the 186R DNA

TABLE 3. Further characterization of the pairs of selected cloned variants

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Susceptibility (IC&lt;sub&gt;50&lt;/sub&gt;, μM)&lt;sup&gt;a&lt;/sup&gt; to:</th>
<th>Virus titers (PFU/ml)</th>
<th>Animal pathogenicity&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>ACV Ganciclovir PAA Primary Secondary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>260 320 47</td>
<td>1 x 10&lt;sup&gt;3&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>1B</td>
<td>3.2  3.6 24</td>
<td>3.1 x 10&lt;sup&gt;3&lt;/sup&gt; 3.2 x 10&lt;sup&gt;3&lt;/sup&gt;  +</td>
<td>+</td>
</tr>
<tr>
<td>2A</td>
<td>1.8  3.5 89</td>
<td>5.7 x 10&lt;sup&gt;3&lt;/sup&gt; 2.0 x 10&lt;sup&gt;3&lt;/sup&gt;  +</td>
<td>+</td>
</tr>
<tr>
<td>2E</td>
<td>1.1  2.6 150</td>
<td>2.8 x 10&lt;sup&gt;3&lt;/sup&gt; 6.2 x 10&lt;sup&gt;3&lt;/sup&gt;  +</td>
<td>+</td>
</tr>
<tr>
<td>3B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>&gt;400 &gt;600 82</td>
<td>1.2 x 10&lt;sup&gt;4&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
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<tr>
<td>3I</td>
<td>140  130 74</td>
<td>5.7 x 10&lt;sup&gt;3&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>4A</td>
<td>120  &gt;400 60</td>
<td>4.0 x 10&lt;sup&gt;3&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>4E</td>
<td>52   90  44</td>
<td>3.2 x 10&lt;sup&gt;3&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>5A</td>
<td>470  410 110</td>
<td>3.5 x 10&lt;sup&gt;3&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>5B</td>
<td>52   &gt;250 64</td>
<td>1.5 x 10&lt;sup&gt;3&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>6C</td>
<td>63   NT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.7 x 10&lt;sup&gt;3&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>6E</td>
<td>1.3  2.0 66</td>
<td>3.5 x 10&lt;sup&gt;3&lt;/sup&gt; 2.6 x 10&lt;sup&gt;3&lt;/sup&gt;  +</td>
<td>+</td>
</tr>
<tr>
<td>7FB</td>
<td>4.2  3.1 87</td>
<td>9.4 x 10&lt;sup&gt;3&lt;/sup&gt; 4.3 x 10&lt;sup&gt;3&lt;/sup&gt;  +</td>
<td>+</td>
</tr>
<tr>
<td>7GA</td>
<td>3.0  NT NT</td>
<td>6.3 x 10&lt;sup&gt;3&lt;/sup&gt; 1.2 x 10&lt;sup&gt;3&lt;/sup&gt;  +</td>
<td>+</td>
</tr>
<tr>
<td>BRY</td>
<td>0.5  0.6 52</td>
<td>3.8 x 10&lt;sup&gt;3&lt;/sup&gt; 1.0 x 10&lt;sup&gt;3&lt;/sup&gt;  +</td>
<td>+</td>
</tr>
<tr>
<td>BRY TK&lt;sup&gt;−&lt;/sup&gt;</td>
<td>51 &gt;80 56</td>
<td>3.0 x 10&lt;sup&gt;3&lt;/sup&gt;  ND&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> IC<sub>50</sub>, 50% inhibitory concentration.
<sup>b</sup> Zosteriform model.
<sup>c</sup> ND, Not detectable.
<sup>d</sup> NT, Not tested.
polymerase to PAA was seen. Shifts were not seen with any enzyme assayed from the selected clones. When ACV-TP was substituted for PAA in the assay, again no changes were seen. The relative susceptibility of these crude enzyme preparations to both ACV-TP and PAA is further evidence that the resistance seen in vitro is due to no or low expression of TK.

**Restriction enzyme analysis.** DNA was extracted from four clones and digested with BamHI, BglII, EcoRI, and KpnI. Analysis of the resulting fragments revealed no significant differences between the viruses. The changes that were seen were minor and mapped to the variable junction and terminal repeat regions. These changes are frequently seen, particularly when a series of isolates from a single patient is analyzed (19).

**Neurovirulence.** The pathogenicity of the seven isolates in mice was determined following intracerebral inoculation. The 50% lethal doses (Table 1) for the ACV-susceptible isolates, isolates 1, 2, 6, and 7, were less than 10 PFU. For the three resistant isolates, isolates 3, 4, and 5, the 50% lethal doses were up to 100-fold greater. These results correlate with other reports, showing that increased resistance to ACV is associated with a reduction in virulence to mice inoculated by this route (14).

**Zosteriform model.** A cutaneous primary infection was induced on the upper flanks of mice and subsequently monitored for up to 7 days. Clinical observations were made, and animals were sacrificed on day 5 for removal of skin samples from primary and secondary sites. Clones susceptible to ACV produced a secondary infection from which virus was readily detected by titration of extracts of excised skin samples in Vero cells (Table 3). Infection with the ACV-resistant clones resulted in only a mild primary infection in mice at the site of inoculation. Studies with a TK deletion mutant in our laboratory showed this mutant to be weakly neurovirulent when inoculated onto the flank. However, although a primary infection was established at the inoculation site, there was no evidence of secondary spread.

**DISCUSSION**

We have reported in this paper the biological and biochemical characteristics of a series of HSV-2 clinical isolates from a severely immunocompromised patient. Few reports exist giving such data on sequential isolates from one patient. Two early disease episodes were controlled with prompt aggressive therapy, but subsequently, during an indolent episode of perianal disease, ACV-resistant virus was isolated. A change in therapy did ameliorate the infection, but the patient remained culture positive until he died. Consistent with other reports (3, 9, 16, 24, 28, 30), resistance may occur in immunocompromised patients who progress to persistent disease, particularly if ACV therapy is delayed or insufficiently aggressive.

The susceptibility data revealed that isolates taken during protracted courses of oral therapy were resistant to the TK-activated compounds ACV and ganciclovir. Studies of enzyme extracts of the parental virus showed that resistance was probably due to an inability of the virus to express TK as measured in assays of thymidine conversion. All viruses remained susceptible to PAA, suggesting that resistance was unlikely to result from an alteration in the viral DNA polymerase. This was confirmed when enzyme extracts were found to be sensitive to both ACV-TP and PAA. Had there been changes in the sensitivity of the enzyme to inhibition by ACV-TP or PAA, this would be indicative of an alteration at the DNA polymerase locus (22).

The nature of the resultant virus populations was further examined by producing a number of plaque purified variants of isolates 1 to 7. Biological and biochemical characterization of selected cloned viruses revealed that pretreatment isolates consisted of a mixture containing approximately one-third TK-deficient viruses. Furthermore, plaque purification of the last resistant isolate showed this to consist of 8 of 19 TK-deficient clones (Table 2). The extended courses of ACV therapy have exerted a selection pressure on this mixed virus population, resulting in all plaque-purified viruses being shown to be TK deficient during the resistant phase. Significantly, disease was later amenable to therapy following the removal of the selection pressure, in that ACV administration was stopped for 3 weeks and topical phosphonoformic acid was administered for 7 days. This initiated a partial restoration of a wild-type population (isolates 6 and 7), which was successfully controlled with high i.v. doses of ACV.
Studies of mice confirmed that the recovered ACV-resistant, TK-deficient viruses had low virulence, failing to invade the peripheral nervous system and subsequently the central nervous system or to establish latent infection following infection of the flank. Furthermore, neurovirulence was decreased by 3 orders of magnitude following intracerebral inoculation. These findings are consistent with those of previous workers, who found that TK-deficient virus is less virulent, less likely to establish a latent infection, and less neurovirulent than wild-type virus (10, 14, 29).

The isolates were confirmed as being HSV-2 by restriction endonuclease digestion. Analysis of the resulting band patterns was indicative of infection by a single strain, but these results should be interpreted with care (19). By comparing a number of isolates from a single patient, it could be seen that there were a number of changes in the restriction patterns but that most changes occurred in the variable terminal and subterminal regions (19). (Unfortunately, the last isolates were unavailable for analysis.)

In conclusion, the initial high-dose i.v. therapy given during the first disease episode successfully ameliorated this HSV infection. When the infection recurred, therapy was delayed, and hence when oral treatment was prescribed, success was limited. Resistant virus was isolated which persisted despite more aggressive i.v. therapy. Subsequent removal of the selection pressure and possibly the introduction of phosphonoformic acid resulted in the reestablishment of a susceptible virus population which responded to high-dose i.v. ACV therapy. Extended periods of ACV therapy, particularly in immunocompromised patients, may lead to treatment failure if resistant virus is selected. However, the correlation between in vitro susceptibility and response to therapy is not always clear (12, 20). With this patient, interruption of ACV therapy and possibly the introduction of a non-TK-activated form of antiviral therapy (phosphonoformic acid) resulted in a further course of i.v. ACV therapy, successfully eliminating the HSV infection. The last isolate recovered at this time was susceptible to ACV.

ACKNOWLEDGMENTS

We thank G. Darby for constructive criticism, V. Parmar for performing the virus susceptibility assays, and P. Ward for typing the manuscript.

LITERATURE CITED


