Characterization of a DNA Polymerase Mutant of Herpes Simplex Virus from a Severely Immunocompromised Patient Receiving Acyclovir

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SUMMARY

A series of herpes simplex virus isolates were recovered from a bone marrow transplant patient who received prolonged acyclovir therapy for indolent herpes simplex mouth and throat ulceration. Of 14 isolates received 10 were resistant to acyclovir and partially resistant to phosphonoacetic acid. Biochemical characterization revealed that resistance was due to an alteration in the virus DNA polymerase. DNA sequence analysis of the polymerase gene of a plaque-purified resistant virus isolate revealed a single nucleotide change when compared with the sequence of the gene of a plaque-purified sensitive isolate. This single base change resulted in a predicted amino acid substitution of Gly to Ser at residue number 841, a putative functional region of the polymerase.

INTRODUCTION

In immunocompromised patients such as marrow allograft recipients or patients with AIDS manifestations of herpesvirus disease are frequently serious and may occasionally be life-threatening. Despite the chronic nature of herpesvirus infections in these patients acyclovir (ACV) treatment has been shown to be effective clinically (Mitchell et al., 1981; Wade et al., 1982; Meyers et al., 1982; Whitley et al., 1984; Shepp et al., 1985). However, the prolonged treatment required for these indolent infections may result in the selection of resistant virus variants (Barry et al., 1985; Collins & Oliver, 1986; Collins, 1988). The frequency of such occurrences cannot be determined, since the sensitivity of virus isolates has only been assessed following poor clinical response, and even this did not always correlate with the appearance of a resistant phenotype (Barry et al., 1985).

In cell culture it has also been shown that prolonged exposure of herpes simplex virus (HSV) to ACV results in the selection of resistant virus. Resistance generally results from mutation in the gene encoding thymidine kinase (TK), resulting most frequently in failure to express active enzyme but occasionally in expression of TK with altered substrate specificity such that it is unable to phosphorylate ACV (Field et al., 1980; Coen & Schaffer, 1980; Schnipper & Crumpacker, 1980; Smith et al., 1980; Furman et al., 1981). Less frequently, variants have been isolated which express altered DNA polymerases with reduced sensitivity to inhibition by ACV triphosphate, the active form of the drug (Field et al., 1980; Coen & Schaffer, 1980; Schnipper & Crumpacker, 1980; Furman et al., 1981; Larder & Darby, 1985).

Experience in the clinical situation appears to parallel that in laboratory studies, in that the majority of resistant isolates characterized to date fail to express active TK. In fact, in isolates of HSV from normal individuals TK-deficient virus can be detected at a frequency of approximately 1/1000 (Smith et al., 1980), and it is likely that on exposure to ACV resistance occurs as a result of selection of these variants. In this paper we present data on a series of ACV-resistant HSV clinical isolates from a severely immunocompromised patient in which resistance was not due to TK deficiency but rather to mutation in the DNA polymerase gene (Parker et al.,...
1987). Plaque-purified variants of the isolates have been characterized biochemically, and nucleotide sequence data from the polymerase gene revealed that the resistant phenotype was due to a single amino acid substitution. This is the first recorded incidence of clinical resistance due to alteration in the virus DNA polymerase.

METHODS

Clinical details. The clinical course and details of the virus isolates are summarized in Fig. 1 and are presented in greater detail by Parker et al. (1987). Briefly, a 34-year-old woman with chronic myeloid leukaemia in accelerated phase underwent bone marrow transplantation in October 1985. Despite prophylactic ACV begun prior to transplantation, mucositis and mouth ulcers became a persistent problem. ACV therapy was increased with little effect and was then interrupted for more than 6 weeks, during which time virus was no longer recovered. Subsequently, lip lesions were successfully ameliorated with ACV cream but the patient responded only slowly to later courses of intravenous ACV. In August 1986 the patient died of an overwhelming pseudomonas chest infection.

Cell culture. Vero cells were used for the production of virus stocks, plaque purification and sensitivity assays and were grown in 10% Glasgow modified minimal essential medium (Gibco) supplemented with 10% newborn calf serum (Gibco).

BHK-21 and BuBHK (TK-deficient) cells were used for the production of enzyme extracts and virus DNA. These were cultured in 10% Glasgow modified minimal essential medium, supplemented with 10% tryptose phosphate broth and 10% newborn calf serum.

Isolation and typing of herpesviruses. Throat swabs submitted in Hank's transport medium (Cruikshank et al., 1975) were grown in tube cultures of human embryo fibroblast (HEF) cells growing in Eagle's medium supplemented with 10% inactivated foetal calf serum (Cruikshank et al., 1975). Virus passaged in Vero cells was typed by indirect immunofluorescence using a polyclonal rabbit anti-HSV type 1 (HSV-1) serum and sheep anti-rabbit antibodies conjugated to fluorescein isothiocyanate (Peutherer et al., 1982). The stained slides were examined using a u.v. incident light microscope (Leitz SM-LUX).

For sensitivity assays virus isolates were amplified by single passage in Vero cells and the resultant virus extracts were divided into samples and stored at −70 °C.

Plaque-purified virus was prepared by picking individual plaques following limiting dilutions of virus stocks in Vero cells under a carboxymethyl cellulose overlay. Two cycles of plaque purification were carried out to ensure homogeneous virus populations. The plaque-purified variants were amplified in Vero cells and working stocks were stored in portions at −70 °C.

The following laboratory strains of HSV-1 were used as controls: SC16 (Hill et al., 1975), DM21, a TK deletion mutant generated from SC16 (S. Kemp et al., unpublished) and TP2.5, an altered DNA polymerase mutant generated from SC16 (Larder & Darby, 1985).

Compounds. ACV was manufactured by Wellcome and was prepared as a 1 mM aqueous stock solution. Phosphonoacetic acid (PAA) was kindly supplied by Dr P. Furman (Burroughs Welcome, U.S.A.) and was prepared as a 10 mM aqueous stock solution. Stock solutions were stored in aliquots at −20 °C and used once only.

Drug sensitivity assays. Drug sensitivities were determined by plaque reduction assays in Vero cells as previously described (Collins et al., 1982), using doubling μM concentrations of ACV and PAA. Sensitivities were expressed as 50% inhibitory concentrations (IC₅₀ values).

TK assay. Viral TK activity was measured by the method described by Klemperer et al. (1967) using extracts from BuBHK cells infected with 5 to 10 p.f.u./cell and incubated for 18 h at 37 °C. Aliquots of enzyme extracts 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). The phosphorylation of thymidine was expressed as pmol converted per 10⁶ cells. Alternatively, [3H]ACV (DuPont) was used as a substrate.

DNA polymerase assay. Virus DNA polymerase activity was assayed at 37 °C by the method described previously by Larder et al. (1983) using activated calf thymus DNA as template. The reaction mixtures contained [3H]dGTP at 5 μM (27-6 μCi/mg) in order to monitor the incorporation of substrate into the template DNA. Inhibition of polymerase activity was expressed as the IC₅₀ (μM).

Restriction enzyme analysis. Virus DNA was isolated from infected BHK cells essentially as described by Pignatti et al. (1979), and digested with BamHI, EcoRI, HindIII or SalI restriction enzymes. Restriction patterns were examined by electrophoresis in 0-8% Tris-acetate–EDTA–agarose gels.

DNA sequencing. The cloning strategy described by Larder et al. (1987) was used to obtain fragments of the DNA polymerase genes for sequencing. Procedures used for ssDNA sequencing have also been published previously (Larder et al., 1987). Briefly, ssDNA was isolated by polyethylene glycol precipitation from M13mp8 clones and inserts were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (1977). A set of 20 oligonucleotides (17-mers) were used as primers (made with a Biosearch 8600 synthesizer) and 35S-labelled dATP. Sequencing gels were run using the buffer gradient system of Biggin et al. (1983).
Characterization of acyclovir resistance

A total of 14 isolations of HSV were made from the patient over an 8 month period (Fig. 1). Isolates were cultured in HEF cells and the virus was typed by indirect immunofluorescence following culture in Vero cells. All isolates were shown to be type 1.

**Sensitivity assays**

The sensitivity of each of the isolates was determined in plaque reduction assays in Vero cells and revealed that isolates 1 and 12 to 14 were sensitive to ACV, with IC₅₀ values in the range of 1.7 to 5.2 µM. Isolates 2 to 11 were resistant to ACV, with IC₅₀ values in the range of 17 to 38 µM (Fig. 2). Those isolates resistant to ACV were found to be resistant or partially resistant to PAA (Fig. 2). The final isolate (14) despite being sensitive to ACV was found to be partially resistant to PAA. The HSV-1 wild-type strain SC16 which was sensitive to ACV and PAA, DM21 which was resistant to ACV but sensitive to PAA and TP2.5 which was resistant to both ACV and PAA, were included as controls in the sensitivity assays (Fig. 2).

**DNA polymerase assays**

The observed cross-resistance of these isolates to ACV and PAA was suggestive of resistance resulting from an alteration in the virus DNA polymerase. To test this, crude enzyme extracts were prepared in BuBHK cells infected with either the resistant isolate 8, the sensitive isolates 1 or 13 and the control viruses SC16 or TP2.5. These extracts were used to compare the sensitivities of the virus-induced DNA polymerases to ACV triphosphate and PAA (Table 1). The IC₅₀ values for both inhibitors obtained for the sensitive virus compared well with the wild-type control virus SC16. However, the DNA polymerase from the resistant virus 8 and the DNA polymerase mutant TP2.5 were five- to 10-fold less sensitive, confirming that resistance was at least in part due to an alteration in the sensitivity of DNA replication to inhibition by ACV triphosphate.

**TK assays**

To examine whether a deficiency in the virus TK contributed to resistance, the ability of enzyme extracts prepared in BuBHK (TK-deficient cells) to phosphorylate [¹⁴C]thymidine was measured. The sensitive isolates 1 and 13 were compared with resistant isolates 4, 8 and 10. SC16
"ff := k ≥ c, <

y_'

\int \frac{1}{x} \, dx = \ln|x| + C

345

\sin x = \frac{2\tan \frac{x}{2}}{1 + \tan^2 \frac{x}{2}}

Virus isolate number Controls

Fig. 2. In vitro sensitivity of the clinical isolates of HSV to ACV and PAA. Included as controls were a wild-type strain of HSV-1, SC16 (S), a TK deletion mutant of SC16, DM21 (D), and a DNA polymerase mutant of SC16, TP2.5 (T). Open columns indicate ACV sensitivity; stippled columns represent PAA sensitivity.

Table 1. Sensitivity of crude extracts of virus DNA polymerase from infected BuBHK cells to ACV triphosphate and PAA

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>ACV triphosphate IC_{50} (μM)</th>
<th>PAA IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>12.0</td>
</tr>
<tr>
<td>8</td>
<td>1.8</td>
<td>71.0</td>
</tr>
<tr>
<td>13</td>
<td>0.18</td>
<td>8.6</td>
</tr>
<tr>
<td>SC16</td>
<td>0.34</td>
<td>6.0</td>
</tr>
<tr>
<td>TP2.5</td>
<td>2.9</td>
<td>56.0</td>
</tr>
</tbody>
</table>

was included as a positive control and DM21 as a negative control. In a 10 min assay all except the negative control, DM21, phosphorylated thymidine efficiently, suggesting that there was no deficiency in the TK activities of any of the resistant isolates. (Fig. 3).

Plaque-purified variants

Isolates 1, 8 and 13 were selected for further investigation by plaque purification. After two cycles of plaque purification approximately 10 clones of virus were obtained from each isolate and sensitivity assays performed (Fig. 4).

Each isolate examined appeared to consist of a relatively homogeneous virus population. Virus purified from isolate 1 was consistently sensitive to ACV (0.7 to 3.3 μM) although they were in the upper end of the range previously observed for type 1 viruses (Collins & Oliver, 1986).
Characterization of acyclovir resistance

Fig. 3. Comparison of the activity of extracts of virus TK from infected BuBHK cells as measured by the conversion of $[^{14}C]$thymidine. Extracts from cells infected with the TK deletion mutant DM21 (⊤) or mock-infected (▼) were used as controls. SC16 (■) was used as a positive control. Isolates 4 (●), 8 (○), 1 (□), 13 (▲) and 10 (△).

Fig. 4. In vitro sensitivity of plaque-purified clones of isolates 1, 8 and 13 to ACV and PAA.
Table 2. In vitro sensitivities and biochemical characterization of selected plaque-purified clones of isolates 1, 8 and 13 compared with the control viruses SC16 and TP2.5

<table>
<thead>
<tr>
<th>Plaque-purified variant</th>
<th>ACV (μM)</th>
<th>PAA (μM)</th>
<th>Ara-A (μM)</th>
<th>Thymidine (μM)</th>
<th>ACV triphosphate (μM)</th>
<th>PAA (μM)</th>
<th>ACV (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-B6</td>
<td>3.3</td>
<td>218</td>
<td>36</td>
<td>0.4</td>
<td>0.16</td>
<td>4.2</td>
<td>+</td>
</tr>
<tr>
<td>1-H2</td>
<td>0.9</td>
<td>119</td>
<td>22</td>
<td>0.1</td>
<td>0.16</td>
<td>3.4</td>
<td>+</td>
</tr>
<tr>
<td>8-E12</td>
<td>1.9</td>
<td>436</td>
<td>35</td>
<td>0.2</td>
<td>0.9</td>
<td>12.5</td>
<td>+</td>
</tr>
<tr>
<td>8-H9</td>
<td>15.5</td>
<td>245</td>
<td>33</td>
<td>0.1</td>
<td>0.36</td>
<td>15.0</td>
<td>+</td>
</tr>
<tr>
<td>13-H5</td>
<td>2.9</td>
<td>90</td>
<td>24</td>
<td>0.1</td>
<td>0.03</td>
<td>3.9</td>
<td>+</td>
</tr>
<tr>
<td>13-B9</td>
<td>4.0</td>
<td>348</td>
<td>47</td>
<td>0.2</td>
<td>0.23</td>
<td>3.7</td>
<td>+</td>
</tr>
<tr>
<td>SC16</td>
<td>0.4</td>
<td>85</td>
<td>48</td>
<td>0.7</td>
<td>0.17</td>
<td>4.4</td>
<td>+</td>
</tr>
<tr>
<td>TP2.5</td>
<td>7.9</td>
<td>1153</td>
<td>NT*</td>
<td>NT</td>
<td>NT</td>
<td>70.0</td>
<td>+ NT</td>
</tr>
</tbody>
</table>

*NT, Not tested.

Viruses purified from isolate 8 were 10 to 30-fold less sensitive to ACV than those from isolate 1 and two- to fourfold less sensitive to PAA, although there was slight variation these values compared well with the uncloned isolate.

The ACV sensitivities of the variants from isolate 13 correlated well with the uncloned isolate, falling in the range of 2.5 to 5.8 μM. They were, however, less sensitive than those of the early sensitive isolate 1. With the exception of 13-B9 the variants were all sensitive to PAA. Interestingly this virus was sensitive to ACV and resembled isolate 14 which was also sensitive to ACV and partially resistant to PAA.

Two plaque-purified variants were selected from isolates 1, 8 and 13 for further characterization, namely 1-B6, 1-H2, 8-E12, 8-H9, 13-H5 and 13-B9, and were compared with the control viruses SC16 and TP2.5. There was no evidence of cross-resistance to adenine 9-β-D-arabinofuranoside (ara-A) or aphidicolin. All maintained a TK-positive phenotype with thymidine and ACV as substrates. In DNA polymerase assays enzyme extracts from sensitive virus were more susceptible to ACV triphosphate and PAA than extracts from resistant virus; however, the differences were less striking than had been previously observed with the parent virus (Table 2).

Nucleotide sequence analysis of DNA polymerase genes

The pattern of drug resistance observed with isolate 8 together with biochemical data described above strongly suggested that this virus had a mutant DNA polymerase. This was investigated further by comparative DNA sequence analysis of the clones 8-H9 and 1-H2.

Initially we confirmed, by restriction enzyme analysis using a number of different enzymes, that the early sensitive isolates 1-H2 and the ACV-resistant isolate, 8-H9, were derived from the same strain (data not shown). Overlapping restriction enzyme DNA fragments (BamHI and EcoRI) spanning the polymerase gene coding region were isolated from 1-H2 and 8-H9 and used for cloning into M13mp8 (see Methods). The entire nucleotide sequence of the polymerase gene was determined, and it was shown that the nucleotide sequence of the ACV-resistant isolate 8-H9 was identical to that of 1-H2 except for a single base transition (G → A) at nucleotide 2521. This change would lead to a predicted amino acid substitution (Gly → Ser) at residue 841 of the polymerase polypeptide.

The polymerase gene of the laboratory wild-type HSV-1 strain (SC16) has been sequenced previously (Larder et al., 1987) and comparison of the polymerase gene of the ACV-sensitive isolate 1-H2 with this laboratory strain revealed seven nucleotide changes leading to four amino acid substitutions. The extent of the sequence conservation observed between these strains is similar to that observed between other wild-type HSV-1 polymerase genes (Gibbs et al., 1985; Quinn & McGeoch, 1985; Knopf, 1987).
DISCUSSION

A number of HSV-1 isolates obtained from a bone marrow transplant patient receiving ACV prophylaxis and subsequent therapy for indolent oral HSV, were found to be resistant to ACV. They were also partially cross-resistant to PAA, with increases in IC₅₀ values of two- to fivefold compared with sensitive virus. Cross-resistance to PAA suggested that there was an alteration in the virus DNA polymerase. Sensitivity assays of virus DNA polymerase to ACV triphosphate and PAA confirmed that enzymes from resistant viruses were up to 10-fold less susceptible to inhibition than those encoded by the sensitive viruses.

There was no evidence for an involvement of TK in the resistance phenotype, as sensitive and resistant isolates were shown to phosphorylate, with equal efficiency, both thymidine and ACV. Subsequent analysis of sensitive and resistant virus populations by plaque autoradiography incorporating [¹²⁵I]iododeoxyctydine confirmed that virus was 100% TK-positive (N. Ellis, personal communication). Sensitivity assays on plaque-purified variants from isolates 1, 8 and 13 provided further evidence that the virus populations did not comprise significant mixtures. However, one of ten of the cloned variants prepared from isolate 13 was partially resistant to PAA but sensitive to ACV. This may go some way to explain the unexpected finding with isolate 14, which remained sensitive to ACV but had become resistant to PAA. This isolate was received some time after isolates 1 to 13 and has yet to be characterized fully.

The biological and biochemical evidence supports the view that resistance in this series of isolates was due to an alteration in the virus-encoded DNA polymerase. Nucleotide sequence analysis of the DNA polymerase gene of one of the resistant variants (8-H9) revealed a single base change (G → A) at nucleotide residue 2521 compared with that of the sensitive variant (1-H2). This change would lead to a predicted amino acid substitution (Gly → Ser) at residue 841. The change observed in 8-H9 was identical to that found previously in a laboratory-generated DNA polymerase mutant (TP2.7) derived from the wild-type SC16 (Larder et al., 1987). In this case it was confirmed by marker rescue analysis that the mutation in TP2.7 conferred resistance to ACV. On the basis of sequence data of a number of DNA polymerase mutants and comparisons with existing sequence analysis of other DNA polymerases a number of putative functional regions of the polymerase polypeptide have been predicted. The substitution observed in 8-H9 and TP2.7 is in region III, which is thought to have a direct role in nucleotide binding. This series of isolates and TP2.7 contrasted with the highly PAA-resistant DNA polymerase mutant TP2.5, which was used in this study as a control DNA polymerase mutant. It is worth noting that although the clone 1-H2 derived from the pre-therapy isolate (1) showed considerable homology with our wild-type strain SC16, there were in all seven nucleotide differences, four of which would be expected to lead to amino acid alterations. None of these changes were in putative functional regions of the polypeptide.

Despite the fact that ACV resistance, both clinical and virological, can emerge as the result of a single base change in the DNA polymerase gene, there appears to be a restricted number of sites at which changes may occur that would result in resistance whilst maintaining a functional polymerase. This constraint could explain why it is relatively difficult to generate ACV-resistant DNA polymerase mutants in the laboratory (Larder & Darby, 1985, 1986), and furthermore why there has only been this one case of clinical resistance to ACV resulting from an altered DNA polymerase reported to date. The clinical significance of such a change is difficult to assess and studies in animal models are under way to determine the pathogenicity of these isolates.

REFERENCES


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