Thymidine Kinase Activity of Ocular Herpes Simplex Isolates Resistant to IUDR Therapy

Martha L. Funderburgh,* James L. Funderburgh,* and John W. Chandler†

Thymidine kinase (TK) activity was examined in extracts of TK-deficient cells infected with low passage isolates of herpes simplex virus type 1 (HSV-1) obtained from patients with herpetic keratitis. TK activity induced by the virus, relative phosphorylation rates of thymidine and iododeoxyuridine (IUDR), and Km values for thymidine and for IUDR were compared for TK induced by the different viral isolates. Four of the five isolates showing IUDR resistance in vitro and in vivo also exhibited alterations in properties of the viral TK. Two induced very low levels of viral TK. Three (including one with reduced TK) had increased Km values for IUDR. A fifth IUDR-resistant isolate induced TK similar to the IUDR-sensitive isolates. The results indicate that modification of viral gene for TK may be responsible for development of clinical resistance to IUDR in many occurrences of herpetic keratitis. Invest Ophthalmol Vis Sci 27:1546–1548, 1986

Five'iododeoxyuridine (IUDR) is an antiviral agent widely used in the treatment of ocular infections of herpes simplex virus (HSV). When used repeatedly for recurrent HSV keratitis, IUDR treatment loses its effectiveness in many cases.1 HSV isolates from patients with IUDR resistant disease show resistance in vitro and, in a rabbit model, in vivo.2 Thus, the virus can acquire properties in vivo which allow it to replicate in the presence of IUDR. IUDR is incorporated into viral DNA in place of thymidine, a process which is initiated by phosphorylation of IUDR by virally thymidine kinase. This enzyme is coded by the viral genome, and differs from cellular TK both in its physical properties and in its ability to phosphorylate IUDR and similar analogues. Normal cells and cells infected with TK-deficient mutants of HSV do not incorporate IUDR into DNA, whereas HSV conditioned to multiply in the presence of IUDR in vitro generally lacks viral TK activity.3,4 Therefore, the antiviral properties of IUDR may depend on the presence of an active viral TK. In this study, we have examined the TK activity of several naturally occurring IUDR resistant isolates. We found that the TK activity of some but not all of these isolates was noticeably altered.

Materials and Methods. Cells: B82, a TK-deficient cell line derived from Earle's mouse L cells, was kindly provided by Dr. James McDougall. FT, a human fetal tonsil fibroblast line, was used for virus isolation, propagation, and titration. Both cell lines were maintained in Eagle’s minimal essential medium supplemented with 10% fetal calf serum and antibiotics, as described previously (MEM-10).5 Virus E115, used as an HSV-1 standard strain, is an isolate from a labial herpetic lesion. Other viruses used are ocular isolates from patients with herpetic keratitis. All patients had been treated with a full course of IUDR (Stoxil; Burroughs-Wellcome, Research Triangle Park, NC) as recommended by the manufacturer. Treatment resistance was determined by the presence of active lesions and culturable virus in the cornea after 1 week of treatment.6 Low passage (six passages or less) viral stocks were grown and titered on FT monolayers in tissue culture tubes in MEM with 2% fetal calf serum (MEM-2). Stocks were stored as aliquots at −70°C, and filtered through a 0.45 μ Millipore Swinnex just before use. HSV isolates used in this study were classified as Type 1 by two methods: immunologic neutralization using guinea pig anti-HSV hyperimmune serum, and comparative plating efficiency on human FT cells and chick embryo fibroblasts.5,6 IUDR resistance in vitro was determined on monolayers of FT cells. The effective dose was defined as the concentration of IUDR required to reduce titer of a viral isolate by two log units.

Enzyme preparation: B82 monolayers, 5 × 10⁶ cells per 60 mm tissue culture dish, were grown in MEM-10 for 30 hr before the addition of the virus. HSV was added in 2 ml MEM-2 at multiplicities of infection (MOI) ranging from 1–5 infecting particles per cell. After 14 hr of incubation at 37°C, monolayers were rinsed with GKN (NaCl, 0.135 M; KCl, 5 mM; glucose, 5 mM), and cells released with 0.2 ml 0.25% trypsin in (GKN + 0.01 M NaHCO₃) for 30 min at 37°C. Released cells were rinsed by centrifugation from MEM-2, then GKN, and then lysed by the addition of cold TK buffer (20 mM Tris, pH 8, 0.15 mM KCl, 25 mM NaF, 1.4 mM 2-mercaptoethanol, 0.5% bovine serum albumin, 1% Nonidet P-40), 0.2 ml per 5 × 10⁶ cells. Cell lysis and subsequent steps were carried out on ice, except as noted. Particulate material was removed from the lysate by centrifugation for 1 min at room temperature in a Beckman (Fullerton, CA) Microfuge (9500 × g), and supernatant was used as a source of TK without further purification.

TK assays: TK activity in cell lysates was assayed at 37°C using 175 μl of the lysate with 75 μl of an assay mix in TK buffer, which provided final concentrations of the following compounds: 2 mM Mg-ATP, 3 mM creatine phosphate, 0.04 mg/ml creatine phosphokinase, and 40 μM thymidine (New England Nuclear, Boston, MA NET-027X) or 40 μM IUDR (Amersham, Arlington Heights, IL TRA,161) each at 20 Ci/m mole. Fifty I samples taken initially, and at three 10-min intervals, were adsorbed onto DEAE cellulose paper circles (DE81-Whatman), and immediately immersed in 90% ethanol. Unphosphorylated nucleoside was removed by three rinses (5 min each) in 90% ethanol, and radioactivity was determined by scintillation
counting of the dried discs. The rate of phosphorylation of tritiated thymidine was found to be linear for all lysates assayed in this manner. TK activity was calculated from linear regression analysis of the four time points for each assay. TK units were defined as picomoles tritiated thymidine phosphorylated per min. Km values for IUDR and thymidine were determined using the same enzyme extracts described above, diluted twofold in TK buffer. Incubation was carried out as described above in a total volume of 250 μl, containing 10 μl enzyme extract, TK buffer, 2 mM Mg-ATP, 3 mM creatine phosphate, 0.04 mg/ml creatine phosphokinase, and thymidine or IUDR at concentrations ranging from 0.2–19 μM. Hundred microliter samples were removed at 0 min, and after 30 min at 37°C, and phosphorylation was measured as described above. Km values were calculated from Lineweaver-Burke plots.

Results. The amount of TK activity in a TK-deficient cell line 14 hr after infection with HSV was found to be a function of the amount of infecting virus added to the culture. Cell lysates from uninfected cultures had no measurable TK. As shown in Figure 1, the amount of TK produced varied among the different viral isolates. Curves similar to those shown in Figure 1 were used to compare the amount of TK induced by eight different ocular viral isolates and one lab strain. These data (Table 1) show considerable variability in the levels of TK induced. Two of the isolates induced levels of TK that were significantly lower than the other seven (P < .01 by t-test). One of these (TFT412) had no detectable activity, and the other (CJ181) induced a TK level 5-50-fold below the other strains. As shown in Table 1, both of these isolates showed resistance to IUDR in vivo and in vitro. Other isolates with IUDR resistance induced TK levels equivalent to or higher than the TK of sensitive viral isolates.

TK induced by the different viral isolates was compared in its ability to utilize thymidine and IUDR as substrates. In the first set of experiments, phosphorylation rates for the two substrates were compared in the presence of high substrate concentrations (40 μM). As shown in Table 2, most of the viral TK from the different isolates utilized IUDR 2–3-fold more rapidly than thymidine. None of the IUDR-resistant isolates was impaired in its ability to phosphorylate IUDR under these conditions. In fact, TK from one resistant isolate (TFT409) phosphorylated IUDR significantly more rapidly than the others at these substrate concentrations.

Usage of the two substrates at lower concentrations was analyzed by the determination of Km values for both thymidine and IUDR in the cell lysates. As shown in Table 2, a significant difference (P < .05) was observed in the Km ratios of three of the isolates. This difference resulted, in each case, from an increase in the Km for IUDR. These TK variants, therefore, require higher concentrations of IUDR to achieve the same phosphorylation rate as average viral TK.

Discussion. Of the five viral isolates examined which had demonstrated resistance to IUDR, four showed significant alteration in properties of the TK induced in infected cells. The most dramatic change was the total lack of TK seen in isolate TFT412. As might be expected, this strain was completely resistant to IUDR in vitro. These results support the generally accepted conclusion that viral TK is required for the incorporation of thymidine analogues into viral DNA. Thus, a viral strain can become resistant by the reduction or elimination of its ability to induce TK. The loss of viral TK has been linked with decreased virulence and the inability to establish latency in the trigeminal ganglia.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>TK Activity (Units/PFU)</th>
<th>IUDR Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJ311</td>
<td>6.50</td>
<td>0</td>
</tr>
<tr>
<td>CJ394</td>
<td>1.11</td>
<td>0</td>
</tr>
<tr>
<td>CJ360</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>El15</td>
<td>3.5</td>
<td>NA</td>
</tr>
<tr>
<td>CJ372</td>
<td>5.62</td>
<td>0</td>
</tr>
<tr>
<td>CJ181</td>
<td>0.18†</td>
<td>+</td>
</tr>
<tr>
<td>TFT407</td>
<td>2.12</td>
<td>+</td>
</tr>
<tr>
<td>TFT409</td>
<td>11.5</td>
<td>+</td>
</tr>
<tr>
<td>TFT412</td>
<td>&lt;0.05‡</td>
<td>+</td>
</tr>
</tbody>
</table>

- *Reported clinical resistance in IUDR. †Indicates resistance.
- ‡IUDR concentration in μg/ml required to reduce viral tier 2 log units.
- ¶Significantly less than the mean (P < .01) by single-sided t-test.
- ‡Below limits of assay.

Fig. 1. Relationship between thymidine kinase and an infectious dose of HSV. The amount of TK induced in cultures of TK-deficient B82 cells was determined for several infectious doses of three different HSV-1 isolates using the assay described in Materials and Methods. The lines represent linear regression curves on the original data. Isolates: TFT409 (open circles), El15 (solid circles), CJ181 (squares).
the virus infecting the cells; and (3) Km values for thy-

creased phosphorylation of IUDR by these isolates is
IUDR in vitro, as well as their clinical resistance to

could explain the partial resistance of these isolates to

laton rate to a great degree; (2) identical concentrations
were probably not affecting the measured phosphory-
laton rate to a great degree; (2) identical concentrations
were measured for each substrate. Three isolates
showed significant increases in apparent Km for IUDR.
Each of these isolates was moderately resistant to IUDR
in vitro, and two had been reported as treatment-re-
sistant clinically. This correlation suggests that TK was
involved in the resistance. Since the Km values were
not obtained with purified enzymes, they may not rep-
resent the true Km of the viral TK; however, the data
are useful for the comparison of viral isolates for the
following reasons: (1) no change in measured Km was
found over a tenfold range in lystate concentration (data
not shown); consequently, inhibitors in the cell lysate
were probably not affecting the measured phosphory-
laton rate to a great degree; (2) identical concentrations
of lystate were used in each assay, so that differences in
measured Km can be attributed only to differences in
the virus infecting the cells; and (3) Km values for thy-
midine did not vary greatly among the isolates and,
therefore, acted as an internal control. The results of
the Km determination showed that extracts of cells
infected with three of the IUDR-resistant isolates
phosphorylate IUDR much more slowly than sensitive
isolates under conditions of limited concentrations of
IUDR. Extrapolating this finding to intact infected cells
could explain the partial resistance of these isolates to
IUDR in vitro, as well as their clinical resistance to
IUDR therapy. The simplest explanation of the de-
creased phosphorylation of IUDR by these isolates is
that it results from an alteration of the viral TK. Other
explanations might be possible as well.

The three isolates with increased Km for IUDR were
sensitive in vitro to IUDR concentrations that are ap-
proximately equal to the Km measured for IUDR. This
level is several orders of magnitude lower than the con-
centration of IUDR in topical medications. This find-
ing suggests that topical application may not provide
continuous presence of IUDR at the low (micromolar)
concentrations required to inhibit these viral isolates,
or that the uptake of IUDR by infected cells may limit
the intracellular concentrations of IUDR.

In contrast to the other four, one isolate which
showed moderate IUDR resistance in vitro appeared
to produce normal TK by the criteria of this study.
Resistance to IUDR may, therefore, arise independ-
ently of alterations in the viral TK. The same con-
clusion was reached in studies with laboratory strains
resistant to another thymidine analogue, acycloguano-
sine.8,9 In conclusion, our data show that alterations
in the viral TK may play a role in the development of
treatment resistance of HSV to topical IUDR treatment
to ocular infections. Alteration of the viral TK, how-
ever, may not be the only mechanism by which this
resistance can arise.

Key words: cornea, herpes simplex virus type 1, 5'-iodode-
oxuridine, thymidine kinase, antiviral drugs, drug resistance

From the Corneal Disease Research Laboratory, Swedish Hospital
Medical Center, Seattle, Washington. *Present address: Department of
Biology, Kansas State University, Manhattan, Kansas. ♠Present ad-
dress: Department of Ophthalmology, University of Wisconsin, 600
Hiland Avenue, Madison, Wisconsin. Supported by NIH Grant EY
02902. Submitted for publication: September 23, 1985. Reprint re-
quests: James L. Funderburgh, Division of Biology, Kansas State
University, Ackert Hall, Manhattan, KS 66506.

References
1. Colemen V, Tsu E, and Jawetz E: Treatment resistance to io-
129:761, 1968.
2. West C, Skahan P, Mok A, and Chandler J: Keratitis in the
rabbit by herpes simplex virus from human corneas. ARVO Ab-
3. Cooper G: Phosphorylation of 3-bromodeoxyctydine in cells
infected with herpes simplex virus. Proc Natl Acad Sci USA 70:
4. Jamison A and Subak-sharpe J: Biochemical studies on the
herpes simplex virus-specified deoxyopyrimidine kinase activity.
5. Wentworth B and French L: Plaque assay of herpes virus hominis
588, 1969.
6. Wentworth B and Zablotsy N: Efficiency of plating on chick
embryo cells and kinetic neutralization of herpes virus hominis
7. Tenser R, Resel S, and Dunstan M: Herpes simplex virus thy-
midine kinase expression in trigeminal ganglion infection; Cor-
relation of enzyme activity with ganglion virus titer and evidence
8. Schnipper L and Crumpacker C: Resistance of herpes simplex
virus to acycloguanosine: Role of viral thymidine kinase and DNA
9. Coen D and Schaffer P: Two distinct loci confer resistance to
acycloguanosine in herpes simplex virus type I. Proc Natl Acad

---

Table 2. Comparison of thymidine
and IUDR as substrates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolate (IUDR/Thym)*</th>
<th>IUDR/Thym</th>
<th>Km (µM)</th>
<th>Km (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJ311</td>
<td>1.75</td>
<td>0.50</td>
<td>0.57</td>
<td>0.87</td>
</tr>
<tr>
<td>CJ394</td>
<td>1.90</td>
<td>0.20</td>
<td>0.41</td>
<td>0.50</td>
</tr>
<tr>
<td>CJ360</td>
<td>2.29</td>
<td>0.56</td>
<td>1.22</td>
<td>0.46</td>
</tr>
<tr>
<td>E115</td>
<td>3.20</td>
<td>0.48</td>
<td>1.36</td>
<td>0.36</td>
</tr>
<tr>
<td>CJ372</td>
<td>1.66</td>
<td>8.35</td>
<td>1.25</td>
<td>6.68‡</td>
</tr>
<tr>
<td>CJ181</td>
<td>1.43</td>
<td>14.80</td>
<td>1.12</td>
<td>13.20‡</td>
</tr>
<tr>
<td>TFT407</td>
<td>1.13</td>
<td>0.36</td>
<td>0.58</td>
<td>0.61</td>
</tr>
<tr>
<td>TFT409</td>
<td>5.48†</td>
<td>1.66</td>
<td>1.34</td>
<td>1.24†</td>
</tr>
</tbody>
</table>

* Phosphorylation rate in the presence of 40 µM substrate.
† P < .05 compared to average of four normal ratios (t-test).
‡ P < .01.

---

*Phosphorylation rate in the presence of 40 µM substrate.
† P < .05 compared to average of four normal ratios (t-test).
‡ P < .01.