Comparison of Thymidine Kinase Activities Induced in Cells Productively Infected with Herpesvirus Saimiri and Herpes Simplex Virus

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SUMMARY

The replication of unselected strains of herpesvirus saimiri (HVS) was sensitive to bromodeoxyuridine and bromovinyldeoxyuridine (BVdU) but insensitive to acycloguanosine (ACG), in contrast to the growth of herpes simplex virus (HSV) which was sensitive to all three analogues. Mutants of HVS resistant to bromodeoxyuridine and BVdU could be selected by growth in the presence of these inhibitors. Productive infections of owl monkey kidney or Vero cell cultures by unselected strains of HVS resulted in increases in a thymidine kinase (TK) activity which was deficient in cells infected with bromodeoxyuridine-resistant mutants of the virus. Induction of the virus enzyme promoted a net increase in the uptake and incorporation of exogenous labelled thymidine in the face of the progressive inhibition of the overall incorporation of [35S]methionine and [3H]uridine into productively infected cells. The TK induced in cells infected with HVS differed from the major activity of uninfected cells and resembled that encoded by HSV in its capacity to phosphorylate iododeoxyuridine and in the sensitivity of all the thymidine phosphorylating activity to competition by BVdU. However, in contrast to the HSV TK, which phosphorylated deoxycytidine and iododeoxycytidine relatively efficiently and was sensitive to ACG, the HVS enzyme did not phosphorylate deoxycytidine or iododeoxycytidine and was insensitive to ACG. Whilst HVS, therefore, shares the characteristic of other members of the herpesvirus group of inducing a novel TK, the properties of the HVS-induced enzyme differ significantly from the enzyme of the prototype herpesvirus, HSV. The properties of the HVS TK are nonetheless sufficiently distinct from those of the uninfected cell to provide a possible basis for selective antiviral chemotherapy based on preferential phosphorylation of nucleoside analogues such as BVdU by infected cells.

INTRODUCTION

The prototype herpesvirus, herpes simplex virus (human herpesvirus 1), has structural genes for a number of non-virion enzyme activities, amongst which thymidine or deoxypyrimidine kinase (Jamieson et al., 1974; Jamieson & Subak-Sharpe, 1974) has received considerable attention as a useful marker for studies of virus genetic and regulatory mechanisms and as an accessible model for studies of eukaryotic gene expression (Honess et al., 1980; Smiley et al., 1980; Wigler et al., 1977; Minson et al., 1978; McKnight & Gavis, 1980). In addition, the ability of this enzyme to phosphorylate deoxycytidine (Jamieson & Subak-Sharpe, 1974), 5-bromo- or 5-iodo-2'-deoxycytidine (Cooper, 1973; Summers & Summers, 1977) are measures of the wide range of phosphate acceptors which form substrates for the virus-coded activity. In recent years a number of anti-herpes compounds have been investigated whose selective antiviral activity is partly or mainly dependent upon their efficient phosphorylation.
in virus-infected as compared to uninfected cells by virtue of the action of the virus thymidine kinase. Thus, the importance of the enzyme as a selective activator of compounds such as acycloguanosine [9-(2-hydroxyethoxymethyl)guanine, acyclovir; Elion et al., 1977; Fyfe et al., 1978; Collins & Bauer, 1979; Field et al., 1980; Darby et al., 1980] and (E)-5-(2-bromovinyl)-2'-deoxyuridine (De Clercq et al., 1979; Allaudeen et al., 1981) has also provided a utilitarian impulse to studies of the virus enzyme.

We have recently begun an investigation of the molecular biology of a very different subgroup of herpesviruses, the so-called simian lymphotropic herpesviruses presently typified by herpesvirus saimiri (HVS) and herpesvirus ateles (HVA). These viruses naturally infect squirrel (Saimiri sciureus) and spider (Ateles sp.) monkeys to give persistent, asymptomatic, infections involving peripheral lymphocytes in a manner reminiscent of the Epstein–Barr virus of man, but with the significant distinction that HVS and HVA are apparently latent in T lymphocytes in contrast to the B lymphocyte specificity of the Epstein–Barr virus. However, in a number of other species of New World monkeys, infection with HVS or HVA results in rapidly fatal lymphoproliferative diseases (for reviews, see Deinhardt, 1973; Fleckenstein, 1979). HVS and HVA give productive infections in cultures of tissues from a number of Old and New World monkeys and are therefore amenable to more convenient analysis than the Epstein–Barr virus of man or similar agents of Old World monkeys. Investigations with HVS provide an opportunity to examine the relationship between the molecular biological properties and the pathogenicity of a lymphotropic herpesvirus and to provide some understanding of the relationships between the diverse branches of the herpesvirus group.

In this paper, we show that HVS and HVA induce the synthesis of a novel thymidine kinase (TK) activity in productively infected cells. The properties of the HVS-induced enzyme are compared with those activities of the uninfected cell and the enzyme encoded by herpes simplex virus.

**METHODS**

**Cells and viruses.** Owl monkey kidney cells (OMK-210; kindly provided by Drs L. V. Melendez and M. D. Daniel, New England Regional Primate Center, Southborough, Boston, Mass., U.S.A. or Professor M. A. Epstein, The Medical School, Bristol, U.K.) and Vero cells (Flow Laboratories) were grown as monolayer cultures at 37 °C in 25 cm² or 75 cm² tissue culture flasks or in rotating 80 oz Winchester bottles in Dulbecco’s modification of Eagle’s medium containing 10% (v/v) newborn calf serum (culture medium). The attenuated derivative of HVS strain 11 (i.e. HVS[11 Att], Schaffer et al., 1975; kindly provided by Drs H. Wolf and G. Bayliss, Pettenkofer Institute, Munich, F.R.G.) or mutants derived from this strain (below) were used for most of the experiments described here. Comparative experiments also employed a phosphonoacetic acid resistant ($P^r$) derivative of a strain presumed to be HVS-SMH1 (Daniel et al., 1975; the isolation and properties of this $P^r$ mutant will be described elsewhere), HVS-HOT (or OMI, Hunt et al., 1973) and HVA strain 73 (the SMH1 strain was provided by Dr H. Wolf; the HOT strain and HVA-73 were from Dr B. Fleckenstein, then at the New England Regional Primate Center). HVS[11 Att] and its derivatives were propagated at low multiplicities of infection (0-01 to 0-5) in cultures of OMK or Vero cells. From each of these cell lines the majority (> 90%) of infectious virus is excreted into the medium and yields of approx. 10 p.f.u./cell after 3 to 5 days incubation at 37 °C were obtained routinely to give virus titres of 1 x 10^7 to 8 x 10^7 p.f.u./ml of medium. Vero cells were not fully permissive for the replication of HVS strains SMH1 and HOT or HVA-73, but these viruses gave yields of about 1 p.f.u./cell (2 x 10^6 to 4 x 10^6 p.f.u./ml) from cultures of OMK cells. A temperature-sensitive mutant (ts 98) of HVS[11 Att] and a bromodeoxyuridine (BrdUrd)-resistant ($B^r$) derivative of this mutant (i.e. ts 98B^r-clone 1)
were used for a number of experiments (see text). Both of these viruses replicated normally at the permissive temperature (34 °C) in either Vero or OMK cell cultures to give yields comparable to the ts + parental strain. Isolation and properties of this and other ts mutants will be described elsewhere. The B' derivative was selected by three consecutive passages of ts 98 at multiplicities of 0.1 p.f.u./cell in OMK cells incubated in the presence of 10 (two passages) or 40 μg/ml (third passage) of BrdUrd. B' virus clones were isolated by plaque purification from the yield of the third passage titrated in the presence of 40 μg/ml BrdUrd and finally plaque-purified in the absence of the analogue. Herpes simplex virus strains used were: HSV-1[HFM] P18cl, HSV-1[HFM/STH2] ts B1, HSV-1[B2006] and HSV-1[F1] and their relevant properties are described elsewhere (Honess & Watson, 1977a; Honess et al., 1980; Dubbs & Kit, 1964; Pereira et al., 1976).

Radiochemicals and nucleoside analogues. L-[35S]methionine (>500 Ci/mmol), [methyl-3H]thymidine (47 Ci/mmol), [5,6-3H]uridine (57 Ci/mmol), deoxy-[U-14C]uridine (0.47 Ci/mmol) and [5-125I]iodo-2'-deoxyuridine (200 to 900 Ci/mmol) were all bought from Amersham International. [5-125I]iodo-2'-deoxyuridine (550 Ci/mmol) was purchased from New England Nuclear. Acycloguanosine [ACG; 9-(2-hydroxyethoxymethyl)guanine, acyclovir, mol. wt. 225] was generously provided by Dr D. J. Bauer and by Dr P. Collins of the Wellcome Research Laboratories, Beckenham, Kent, U.K.; bromovinyldeoxyuridine [BVdU; (E)-5-(2-bromovinyl)-2'-deoxyuridine; mol. wt. 332] was a gift from Dr E. de Clerq of the Rega Institute for Medical Research, Belgium via Dr H. Field. Bromodeoxyuridine (BrdUrd; 5-bromo-2'-deoxyuridine; mol. wt. 307.4) was obtained from Sigma, London.

Conditions for high multiplicity infections and labelling infected cultures with radioactive precursors. Monolayer cultures of OMK or Vero cells (2 × 10⁶ to 4 × 10⁶ cells per 25 cm² flask; 1 × 10⁷ to 2 × 10⁷ cells per 75 cm² flask) were inoculated with 2 to 10 p.f.u./cell of HVS or HSV strains in volumes of 1 (25 cm² flasks) or 5 to 10 ml (75 cm² flasks) and incubated at 37 °C (or 34 °C for ts viruses) with continuous agitation for 1 to 2 h. Thereafter, inocula were decanted and replaced with culture medium and the cultures reincubated. Unlabelled mock-infected cultures or cultures infected with HVS were removed after 48 to 60 h incubation for the preparation of enzyme extracts and cultures infected with HSV-1 after 12 h. Cultures were labelled at times and for intervals given in the text with L-[35S]methionine (10 μCi/ml in medium containing 1/10 the concentration of unlabelled methionine of Dulbecco's modification of Eagle's medium) or with [3H]thymidine (15 μCi/ml), [3H]uridine (15 μCi/ml), and [125I]iododeoxyuridine (3 μCi/ml) in culture medium with 2% (v/v) calf serum. At the end of the required period of infection or labelling, cultures were washed two or three times with ice-cold phosphate-buffered saline. Extracts for enzyme assays were prepared by ultrasonic disruption of washed cell suspensions at a concentration of 0.2 × 10⁷ to 1.0 × 10⁷ cells/ml in a buffer containing 0.02 M-tris–HCl pH 7.8, 1 mM-β-mercaptoethanol, and were then made to 50% (v/v) glycerol. Extracts prepared in this way could be stored at -20 °C for 2 to 4 weeks with little loss of enzyme activity.

Labelled cultures were disrupted by ultrasonic oscillation in distilled water and were then processed for measurements of trichloroacetic acid (TCA)-insoluble or methanol-soluble and insoluble radioactivity. Aliquots were made to 10% (w/v) TCA at +4 °C and after incubation for 30 min the resulting precipitates were filtered on to glass-fibre discs (Whatman GF/C, 2.5 cm discs). The precipitates were washed extensively in 5% (w/v) TCA and then with 95% (v/v) ethanol and finally dried and placed in toluene-based scintillation fluid. Methanol-soluble and -insoluble fractions were prepared by making aliquots of infected cells to 66% (v/v) methanol and incubating at -20 °C overnight. Soluble and insoluble fractions were then separated by sedimentation (10000 rev/min for 5 min) and aliquots of methanol-soluble fractions dried on to glass-fibre discs. Methanol-insoluble precipitates were
resuspended by sonication in water and aliquots dried on to glass-fibre discs and the discs placed in toluene-based scintillation fluid. Radioactivity was measured by liquid scintillation spectrophotometry in a Beckman LS 7000.

**Nucleoside kinase assays.** Preliminary experiments with HVS-infected and uninfected cell extracts showed that thymidine kinase activities in crude extracts had rather broad pH optima in the range pH 7.5 to 8.5 in tris–HCl buffers and that tris–HCl buffers in this range gave higher enzyme activities than phosphate buffers over the same pH range. The standard reaction mixture adopted was, therefore, 0.2 M-tris–HCl pH 8.2, 5 mM-MgCl₂, 5 mM-ATP and either 0.2 to 0.32 μM-[3H]thymidine or 4.4 × 10⁻³ μM-[125I]iododeoxyuridine or 3.6 × 10⁻³ μM-[125I]iododeoxycytidine as phosphate acceptors with a total vol. of 0.1 ml per assay. In each series of assays, specific enzyme activities were determined in a dose-response with extracts of known cell and protein concentration (see results). Assays were incubated for 10 min at 37 °C and reactions terminated by immersion in a boiling water bath. Aliquots of the reaction mixture were then dried on to DEAE-cellulose paper (Whatman DE 81) and phosphorylated and unphosphorylated products and reactants separated by 3 × 10 min washes with 1 mM-ammonium formate at pH 3.6 and the radioactivity bound to the DEAE-cellulose paper was measured by liquid scintillation spectrophotometry. Analyses of reaction mixtures by paper chromatography [samples applied to Whatman No. 1 paper and separated by downward development with butan-1-ol : glacial acetic acid : water (2 : 1 : 1) until the solvent front had travelled 38 to 40 cm] showed that the reaction was linear with time and the amount of enzyme extract until >80% of the acceptor nucleoside had been phosphorylated by extracts of infected cells and that the major product under these conditions was the corresponding monophosphate. However, only 30 to 40% of the monophosphate formed was retained by the DEAE-cellulose paper under conditions required to remove ≥99% of unphosphorylated nucleosides. The fraction of monophosphate retained was reproducible and the data given are not corrected for the resulting underestimation of real enzyme activities. Measurements of enzyme activities by complete chromatographic analyses of reaction mixtures (above) showed that relative measures of activity were estimated reliably by the measurements of counts bound to DEAE-cellulose paper.

**Infectivity titrations and plaque-reduction tests.** Infectivity titrations and plaque-reduction tests were performed on monolayers of OMK cells in 25 cm² flats which were infected with a range of appropriate virus dilutions and thereafter incubated for 2 to 3 days (HSV-1) or 6 to 10 days (HVS and HVA) with an overlay consisting of culture medium with 2% (v/v) calf serum made to 0.5% carboxymethylcellulose and with the addition of an appropriate range of drug concentrations (see text). Cultures containing BVdU were incubated in the dark. After incubation, plates were fixed with 10% (v/v) formol-saline, stained with crystal violet and plaques enumerated with the aid of a dissecting microscope.

**Protein determinations.** Protein measurements were made by the method of Lowry et al. (1951) with bovine serum albumin as the calibrating standard.

**RESULTS**

**Sensitivity of virus growth and plaque formation to nucleoside analogues**

The growth of herpes simplex viruses is normally sensitive to a number of deoxynucleoside analogues. The selective antiviral activity of these analogues is conferred by their phosphorylation via the virus-coded thymidine kinase and by their incorporation into DNA by the virus-coded DNA polymerase activity (Introduction). Viruses selected for resistance to such analogues therefore encode a thymidine kinase of reduced (Dubbs & Kit, 1964; Jamieson et al., 1974; Smiley et al., 1980) or altered activity (Darby et al., 1981) or a modified form of the virus DNA polymerase (Coen & Schaffer, 1980; Schnipper &
Table 1. Sensitivity of virus plaque formation on owl monkey kidney cells by unselected and BrdUrd-resistant variants of herpes simplex virus and herpesvirus saimiri to BrdUrd, BVdU and ACG

<table>
<thead>
<tr>
<th>Virus [strain]</th>
<th>Variant (relevant phenotype)</th>
<th>% of control plaque counts observed in the presence of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BrdUrd (130 μM)</td>
</tr>
<tr>
<td>1. HSV-I[HFEI]</td>
<td>P18cl (ts+ P+ TK+)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2. HSV-1[STH2]</td>
<td>ts B1 (ts P+ TK+)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>3. HSV-1[B2006]</td>
<td>(ts+ P+ TK-)</td>
<td>90</td>
</tr>
<tr>
<td>4. HSV-1[F1]</td>
<td>(ts+ P+ TK-)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5. HVS[11 Att]</td>
<td>(ts+ P+ TK+)</td>
<td>0-01–0-04‡</td>
</tr>
<tr>
<td>6. HVS[11 Att]</td>
<td>ts 98 (ts P+ TK+)</td>
<td>0-03–0-43‡</td>
</tr>
<tr>
<td>8. HVS[SMMH1]</td>
<td>P-cl (ts+ P+ TK+)</td>
<td>0-02</td>
</tr>
</tbody>
</table>

* Minority variants; large plaques survive to >44 μM.
† ND, Not done.
‡ Ranges of observed values are larger than the random errors in these measurements and are attributed to variations in the capacity of uninfected TK+ cells to phosphorylate BrdUrd over the time required for the HVS plaque assay.

Crumpacker, 1980; Crumpacker et al., 1980). Preliminary experiments showed that the growth of a number of isolates of HVS and of HVA-73 was sensitive to BrdUrd but that BrdUrd-resistant variants of these viruses could be selected by growth in the presence of the analogue. Table 1 summarizes results from a number of experiments comparing the sensitivity of plaque formation by unselected and BrdUrd-resistant mutants of HSV-1 and HVS to deoxynucleoside analogues. As expected, TK+ isolates of HSV were sensitive to BrdUrd, BVdU and ACG, although a relatively high frequency (1 to 2%) of resistant variants was present in stocks of a number of independent HSV-1 strains (Table 1, lines 1, 2 and 4). The TK- mutant, HSV-I[B2006], was essentially unaffected by relatively high concentrations of all three analogues even when assayed on the TK+ cell lines used in these experiments (OMK cells). Unselected strains of HVS or strains selected for resistance to phosphonoacetic acid were sensitive to BrdUrd and BVdU, but were highly resistant to ACG. The BrdUrd-resistant mutant (HVS[11 Att] ts 98B') was resistant to all three analogues (Table 1, line 7). The apparently greater sensitivity of HVS strains to BrdUrd and the variable plating efficiency of the BrdUrd-resistant strains were attributable to the extended times required for the HVS plaque assay (6 to 10 days) relative to HSV (2 to 3 days) and the inefficient but significant phosphorylation of BrdUrd by uninfected TK+ cells. The BrdUrd-resistant strain of HVS was completely resistant to BVdU under the same conditions of assay.

Increased thymidine uptake and incorporation into cells infected with HVS

Infection of OMK or Vero cells at high multiplicities with HVS results in a productive cycle of virus growth of some 40 to 60 h duration at 37 °C. During the course of such productive infections cellular protein synthesis is inhibited and a progression of structural and non-structural virus-specified polypeptides are made. [We have given preliminary accounts of this process (O'Hare et al., 4th and 5th Herpesvirus Workshops, Cold Spring Harbor, 1979 and 1980) and we will give more detailed descriptions elsewhere (R. W. Honess, R. E. Randall & P. O'Hare, unpublished results; P. O'Hare, R. W. Honess & R. E. Randall, unpublished results)). In Fig. 1(b) we show a typical example of measurements of overall protein synthesis after infection and mock infection of OMK cell monolayers to illustrate the
Fig. 1. Changes in the uptake and incorporation of $^{3}H$thymidine and the incorporation of $^{35}S$methionine into herpesvirus saimiri-infected and mock-infected owl monkey kidney cell cultures. Replicate monolayers of OMK cells were mock-infected (○, O) or infected with 10 p.f.u./cell of HVS[11 Att] (▲, △) and incubated at 37 °C. At intervals after infection and mock infection, duplicate cultures were labelled for 3-h periods with 20 µCi/ml $^{3}H$thymidine (a) or for 9-h periods with 10 µCi/ml $^{35}S$methionine (b). After each labelling period cultures were removed, washed thoroughly with ice-cold phosphate-buffered saline, and the uptake and incorporation of $^{3}H$thymidine (a) into methanol-soluble (O, △) and methanol-insoluble (○, ▲) fractions and $^{35}S$methionine (b) into TCA-precipitable material was measured. Data points are mean values from duplicate cultures and are plotted at the midpoints of each labelling period.

Table 2. Incorporation of labelled precursors into herpesvirus saimiri-infected and uninfected OMK cell cultures

<table>
<thead>
<tr>
<th>Sample</th>
<th>[35S]methionine</th>
<th>[3H]uridine</th>
<th>[3H]thymidine</th>
<th>[125I]iododeoxyuridine</th>
<th>U/M</th>
<th>T/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected OMK cells</td>
<td>280</td>
<td>323</td>
<td>127</td>
<td>3</td>
<td>1-17</td>
<td>0-46</td>
</tr>
<tr>
<td>HVS[11 Att]-infected OMK cells</td>
<td>16</td>
<td>34</td>
<td>163</td>
<td>1-6</td>
<td>2-1</td>
<td>10-1</td>
</tr>
</tbody>
</table>

* Cultures of mock-infected OMK cells and OMK cells infected with 5 p.f.u./cell of HVS[11 Att] were labelled from 30 to 80 h at 37 °C with the indicated precursors and thereafter processed for measurements of incorporation into TCA-precipitable materials.

† Ratios of incorporation normalized to a value of 1-0 for uninfected cells are given in parentheses.

gradual decline in overall protein synthesis characteristic of infected cultures. Also shown in Fig. 1 (a) are measures of the uptake (methanol-soluble pools) and incorporation (methanol-insoluble fraction) of $^{3}H$thymidine into infected and mock-infected cultures which changed in a very different manner in response to infection. Both uptake and incorporation of $^{3}H$thymidine were markedly stimulated in infected cultures at times when overall protein synthesis was declining. Two further experiments showed that this stimulation was specific and did not occur in cells infected with a BrdUrd-resistant mutant of HVS.

In the first experiment, incorporation of $^{3}H$thymidine and $^{125}I$iododeoxyuridine was
Herpesvirus thymidine kinases

Fig. 2. Uptake of [3H]thymidine into the methanol-soluble fractions of mock-infected OMK cells (O) and OMK cells infected with 4 to 5 p.f.u./cell of HVS[11 Att] (△) and with 5 p.f.u./cell of a BrdUrd-resistant derivative of HVS[11 Att] (▲, ts 98B*). Infected and mock-infected cultures were incubated at 34 °C; other experimental details are as described in the legend to Fig. 1.

compared with that of [35S]methionine and [3H]uridine into mock-infected cultures and cultures at late times after infection with HVS. The results (Table 2) showed that the inhibition of overall incorporation seen with [35S]methionine in infected cultures was also observed with [3H]uridine but that [3H]thymidine incorporation was increased in infected cultures and [125I]iododeoxyuridine incorporation did not decrease in proportion to the inhibition of methionine or uridine incorporation. Thus, over this interval, the incorporation of thymidine relative to methionine was increased some 22-fold in infected cultures over that observed in mock-infected cells whereas the relative incorporation of uridine only changed by a factor of about 1-8.

In the second experiment, uptake of [3H]thymidine into the methanol-soluble pools of uninfected OMK cells was compared with that observed after infection with an unselected virus strain, HVS[11 Att], and a BrdUrd-resistant mutant isolated from this strain. The results from this experiment (Fig. 2) showed that the virus-induced stimulation of thymidine uptake did not occur in cells infected with the BrdUrd-resistant mutant. Measures of the overall rates of protein synthesis and an analysis of the time course of virus-specific protein synthesis into cultures infected with the wild-type virus and the BrdUrd-resistant mutant showed that rates of overall and virus-specific protein synthesis changed in a very similar fashion in the two sets of cultures under the permissive conditions used (results not shown).

The experiments described above were all performed with OMK cell cultures which incorporate [3H]thymidine relatively inefficiently after mock infection and thus show a net stimulation after infection. Uninfected Vero cultures incorporate exogenous labelled thymidine much more efficiently than OMK cell cultures and a biphasic response of inhibition followed by stimulation of [3H]thymidine incorporation is observed in infected cultures (not shown).

Measurements of thymidine and iododeoxyuridine kinase activities in extracts of cells infected with BrdUrd-sensitive and -resistant variants of HVS[11 Att]

Direct measurements of kinase activities at periods after infection and mock infection readily demonstrated the induction of an activity in infected cultures which phosphorylated
20
40 60 80
Time post-infection (h)

\[ ^{3} \text{H ct/min/10}^{6} \text{ cells} \times 10^{-3} \]

Fig. 3. Induction of (a) \[^{3} \text{H} \]thymidine and (b) \[^{125} \text{I} \]iododeoxyuridine kinase activities in OMK cells after infection with HVS[11 Att] (●) and after mock infection (○). Replicate monolayers (2 × 10⁶ cells/monolayer) were infected with 5 p.f.u./cell of HVS[11 Att] or were mock-infected and incubated at 37 °C. Duplicate cultures were removed at intervals after infection or mock infection and assayed for their content of (a) thymidine and (b) iododeoxyuridine kinase activities (see Methods; \[^{125} \text{I} \]iododeoxyuridine, 200 Ci/mmol).

\[ ^{3} \text{H ct/min/assay} \times 10^{-4} \]

\[ ^{125} \text{I ct/min/assay} \times 10^{-6} \]

Fig. 4. Relative specific activities of (a) \[^{3} \text{H} \]thymidine and (b) \[^{125} \text{I} \]iododeoxyuridine kinase activities in extracts made from mock-infected OMK cells (○) and OMK cells 48 h after infection with 2 to 4 p.f.u./cell of HVS[11 Att] ts 98 (●), and a BrdUrd-resistant variant, ts 98Br, of this ts mutant (○). Cultures were incubated at the permissive temperature (34 °C) from the time of infection or mock infection until 48 h ([^{125} \text{I} \]iododeoxyuridine, 900 Ci/mmol).

both thymidine and iododeoxyuridine in in vitro assays (Fig. 3a, b). The enzyme activity reached a peak at 40 to 60 h after infection with HVS[11 Att] and similar increases were observed in Vero cells infected with HVS[11 Att] and in OMK cell cultures infected with HVS strains SMH1 and HOT and with HVA strain 73. Increases in the enzyme activities in infected cells were prevented by the addition of 5 µg/ml actinomycin D to cultures at the time of infection (results not shown). More significantly, the kinase activity was greatly reduced in cells infected with BrdUrd-resistant mutants of HVS. An experiment comparing the specific enzyme activities of extracts from uninfected OMK cells and OMK cells 48 h after infection with HVS[11 Att] ts 98 and HVS[11 Att] ts 98Br at the permissive temperature is illustrated in Fig. 4(a, b). With \[^{3} \text{H} \]thymidine as the phosphate acceptor the relative specific activities of
## Table 3. Thymidine (T), iododeoxyuridine (IdUrd) and iododeoxycytidine (IdC) kinase activities in extracts of uninfected OMK and Vero cells and cells infected with herpesvirus saimiri and herpes simplex virus

<table>
<thead>
<tr>
<th>Extract</th>
<th>Kinase activity* observed with phosphate acceptor as</th>
<th>Relative efficiency of phosphorylation (mol/mol)$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[${}^3$H]T</td>
<td>[${}^{125}$I]IdUrd</td>
</tr>
<tr>
<td>Uninfected cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMK</td>
<td>2.9</td>
<td>1.04</td>
</tr>
<tr>
<td>Vero</td>
<td>4.2</td>
<td>2.4</td>
</tr>
<tr>
<td>HVS[11 Att]-infected cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMK</td>
<td>14.0</td>
<td>53.4</td>
</tr>
<tr>
<td>Vero</td>
<td>60.0</td>
<td>200</td>
</tr>
<tr>
<td>HSV-1-infected cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMK</td>
<td>90.0</td>
<td>275</td>
</tr>
<tr>
<td>Vero</td>
<td>360</td>
<td>1200</td>
</tr>
</tbody>
</table>

* $x \times 10^5 = \text{ct/min phosphorylated per mg protein per 10 min.}$

$^\dagger$ $y \times 10^{-2} = \text{mol phosphorylated per mol thymidine.}$

$^\ddagger$ ND, Not done.

extracts of uninfected cells, cells infected with ts 98 and infected with its BrdUrd-resistant derivative were 1:25.7:3.2 (Fig. 4a) and the corresponding figures for activities in the same extracts measured with [${}^{125}$I]iododeoxyuridine as phosphate acceptor were 1:200:5.3 (Fig. 4b). This experiment illustrates more clearly a result which was also shown by the experiment in Fig. 3, i.e. the enzyme induced in HVS-infected cells phosphorylates iododeoxyuridine more efficiently relative to thymidine than does the activity from the uninfected cell. In addition, it is clear that the BrdUrd-resistant mutant is grossly defective in the production of an active virus enzyme and that the residual activity in extracts of cells infected with the mutant virus has an altered capacity to phosphorylate iododeoxyuridine relative to its ability to phosphorylate thymidine.

### Properties of virus and cellular kinase activities

Two further series of experiments were undertaken to examine the distinguishing characteristics of the HSV thymidine kinase and to compare its properties with those of the HSV-1 enzyme. Enzyme extracts from uninfected OMK and Vero cells and cells taken 48 h after infection with HVS[11 Att] or 12 h after infection with HSV-1 [HFEM] P18cl were first assayed for their content of enzymes capable of phosphorylating thymidine, iododeoxyuridine and iododeoxycytidine. The results from these experiments are shown in Table 3. With thymidine as the acceptor nucleoside, cells infected with HVS had specific enzyme activities 5- to 15-fold higher than extracts of uninfected cells and HSV-infected cells had specific activities 30- to 90-fold those of the uninfected cell. Extracts from cells infected with either HSV or HVS showed proportionately much greater increases in their capacity to phosphorylate iododeoxyuridine than thymidine relative to uninfected cells (50- to 90-fold for HSV-infected cells; 300 to 500-fold for HSV-1-infected cells). However, whilst the HSV-1 enzyme also phosphorylated iododeoxycytidine with much higher efficiency than the enzyme from uninfected cells, the HVS-induced activity appeared to have little or no ability to phosphorylate iododeoxycytidine (Table 3, note relative measures of phosphorylation efficiencies). In similar measurements with [${}^{14}$C]deoxycytidine as phosphate acceptor (4.3 $\mu$m) extracts from HSV-infected cells showed no significant increase in kinase activity over extracts from mock-infected cells (results not shown). Enzyme activities in extracts of uninfected cells and cells infected with HVS[11 Att] or HSV-1 responded differently to the addition of unlabelled thymidine, reflecting differences in their $K_m$ for this substrate. The HSV-1 TK was the most sensitive of the three, with the addition of 5 $\mu$m unlabelled thymidine
Fig. 5. Effects of acycloguanosine on the in vitro phosphorylation of [3H]thymidine (0.21 µM) by enzyme extracts from uninfected Vero cells (O), Vero cells 48 h after infection with 5 p.f.u./cell HVS[11 Att] (●) and extracts of Vero cells 12 h after infection with 5 p.f.u./cell HSV-1[HFEM] assayed before (△) and after (▲) the addition of unlabelled thymidine at a concentration of 5 µM. The specific enzyme activities of extracts in the absence of acycloguanosine and unlabelled thymidine were: 1.26 × 10^6 (uninfected cells); 25 × 10^6 (HVS-infected cells); 120 × 10^6 (HSV-1-infected cells) cts/min phosphorylated per 10^7 cells in a 10 min assay.

Fig. 6. Effects of bromovinyldeoxyuridine on the in vitro phosphorylation of [3H]thymidine (0.21 µM) by enzyme extracts from uninfected Vero cells (O), Vero cells 48 h after infection with 5 p.f.u./cell HVS[11 Att] (●), and extracts of Vero cells 12 h after infection with 5 p.f.u./cell HSV-1[HFEM] (△). Enzyme extracts were as for Fig. 5.

reducing the phosphorylation of 0.21 µM labelled thymidine to about 4% of the control value. Corresponding figures for the activities from uninfected cells and cells infected with HVS[11 Att] were 18 to 30% and 15 to 20% respectively. However, very high concentrations of ACG had no effect on the TK activities of uninfected cells or cells infected with HVS. The HSV-1-induced activity was sensitive to ACG but the analogue was a very inefficient competitor compared to thymidine (less than 1 µM unlabelled thymidine reduced the activity with 0.21 µM-[3H]thymidine to 50%, whereas 250 µM-ACG was required to achieve this degree of inhibition). The competitive action of ACG concentrations up to 500 µM was entirely abolished by the addition of 5 µM unlabelled thymidine (Fig. 5). BVdU inhibited both the HSV-1- and the HVS-induced enzymes with high efficiency comparable to that observed with thymidine. The uninfected cell TK activity was composed of a relatively sensitive fraction (about 50%) and a residual activity which was entirely unaffected by very high concentrations of BVdU. The minor fraction of BVdU-resistant TK activity in HVS-infected cells (about 8 to 10%) was due in part to the contribution of this residual resistant activity from the uninfected cell (Fig. 6).

**DISCUSSION**

In this paper we have shown that infections of both OMK and Vero cell cultures with a number of isolates of HVS and one isolate of HVA result in the induction of a novel thymidine kinase activity. The appearance of this activity results in a dramatic increase in the ability of infected cells to incorporate exogenous thymidine and renders virus replication sensitive to BrdUrd and BVdU, but does not confer sensitivity to ACG. Variants of the virus selected for resistance to BrdUrd are also resistant to BVdU and are deficient in the induction of the virus enzyme. In vitro, crude preparations of the HVS enzyme are sensitive to BVdU but insensitive to ACG, unlike the herpes simplex TK activity which is inhibited by both
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compounds. The HVS-induced enzyme differs from that of the uninfected cell in its relatively efficient phosphorylation of iododeoxyuridine but, unlike the herpes simplex enzyme, the HVS-induced TK is not active as a deoxycytidine or iododeoxycytidine kinase.

The HVS-induced TK activity should be of value as a convenient marker for virus gene expression in productively and non-productively infected cells and mutations in the structural gene for the enzyme will assist genetic studies with the virus. The sensitivity of virus growth to analogues such as BVdU as a result of the properties of the kinase further suggests such compounds should prove useful in efforts to modify the fatal virus-induced disease in marmosets. Attempts to demonstrate a virus-specified TK in cells infected with Epstein–Barr virus have given inconclusive results, despite evidence for modifications to deoxynucleoside metabolism in such cells (Hampar et al., 1972; Chen et al., 1978; Colby et al., 1981), but the present results clearly show that a TK-defective phenotype is not a general property of lymphotropic herpesviruses of primates. Given the numerous precedents for a TK activity amongst other members of the group (e.g. Leung et al., 1975; Dobersen et al., 1976; Allen et al., 1979) an analysis of the extent to which these TK genes are homologous in structure and genome location would be of some interest. The complete sequence of the structural gene for the HSV-1 enzyme has been determined by a number of groups (e.g. McKnight, 1980) and similar data for other wild-type and mutant herpesviruses should allow useful structure–activity correlations and some deductions on the evolutionary pathways followed by the enzyme. For example, we suggest that the ability of the enzyme to phosphorylate deoxycytidine may be correlated with its sensitivity to acycloguanosine and is a property of enzymes from herpes simplex, varicella-zoster (Crumpacker et al., 1979; Biron & Elion, 1980), B virus (Boulter, 1980) and probably of other members of the herpes simplex virus neuroseron such as herpesvirus tamarinus and SA8 (Honess & Watson, 1977b). Conversely, viruses such as pseudorabies (Darby et al., 1980), HVS and possibly equine abortion and the herpesvirus of turkeys which encode a TK devoid of deoxycytidine kinase activity are, or may be, relatively resistant to acycloguanosine. The series of herpesvirus TK genes could provide an unusual opportunity to correlate evolutionary changes in enzyme primary structure with resulting modifications in their interactions with substrates and substrate analogues.

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