Properties of Purified Enzymes Induced by Pathogenic Drug-resistant Mutants of Herpes Simplex Virus

EVIDENCE FOR VIRUS VARIANTS EXPRESSING NORMAL DNA POLYMERASE AND ALTERED THYMIDINE KINASE*

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The DNA polymerases and thymidine kinases induced by three drug-resistant mutants of herpes simplex virus type 1 (S1, Tr7, and B3) and their common parent strain, SC16, have been purified and their properties compared. No significant differences were seen in the affinities of the polymerases for TTP and dGTP, or for the triphosphates of 9-(2-hydroxyethyloxymethyl)guanine (acyclovir) or (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVdU) (drugs used in their isolation). In contrast all three mutants induced abnormal thymidine kinases. Those induced by the acyclovir-resistant mutants, S1 and Tr7, showed reduced affinities for thymidine, acyclovir, and also BVdU. Thymidine kinase induced by the BVdU-resistant mutant B3 showed reduced affinity for BVdU, but its affinities for thymidine and acyclovir were similar to those of the wild type enzyme. Thus, it appears that these variants of herpes simplex virus express altered thymidine kinases with impaired ability to phosphorylate particular nucleoside analogue drugs and these characteristics probably account for the drug resistance of the viruses. This strategy for resistance is important as it may result in variants with undiminished pathogenicity.

Many nucleoside analogues inhibit replication of herpes simplex virus (for review see Refs. 1 and 2) and the most effective of them appear to share a common mechanism of action involving activities of two virus-coded enzymes, thymidine kinase and DNA polymerase. It is the triphosphate derivatives of these drugs which interact with virus-specific DNA polymerase to disrupt DNA synthesis (3–7). However, significant activation to the triphosphate can only occur in infected cells as the first stage in this process, formation of the monophosphate, is performed by virus-specific thymidine kinase (3, 8–10).

The involvement of these two separate virus-coded functions in the action of these compounds is confirmed by the observation that the virus can acquire resistance to them by mutation at either genetic locus. This has been demonstrated

with viruses resistant to the drug 9-(2-hydroxyethoxymethyl)guanine) (11–13). Most resistant isolates of HSV[1] obtained following growth of virus in the presence of this drug fail to induce significant levels of thymidine kinase (12–14). Such mutants, with a TK⁻ phenotype, are viable only because thymidine kinase is a nonessential virus function in most tissue culture systems (15, 16), and many reports have now shown that these mutants lack pathogenicity when tested in animals (17–20). Mutants with alterations in DNA polymerase appear to arise with much lower frequency but this may be explained by the constraints on mutation in that gene. Unlike thymidine kinase, DNA polymerase is essential for virus replication and for virus to be viable a mutant DNA polymerase must be capable of performing its normal functions.

Although most acyclovir-resistant mutants fail to express thymidine kinase we have recently shown that more subtle changes in this enzyme, analogous to the substrate specificity changes observed with DNA polymerase, may confer resistance (21, 22). These observations have suggested a further strategy by which HSV can acquire resistance to nucleoside analogues and yet maintain a TK⁺ phenotype, a strategy which may be particularly important since these viruses, unlike TK⁻ variants, appear to retain pathogenicity (18, 21).

In this paper we describe detailed studies on the purified DNA polymerases and thymidine kinases induced by three pathogenic drug-resistant viruses isolated from the same parent HSV-1 strain (SC16). We show in each case that resistance is due to expression of altered thymidine kinase, and the implications of this finding are discussed in relation to the normal function of this enzyme.

MATERIALS AND METHODS

Cells and Tissue Culture—The cells used were baby hamster kidney cells BHK-21; BUdR-resistant BHK cells, which express no cellular thymidine kinase; and BHK TK1 cells, which were made by biochemical transformation of BU-BHK cells to TK⁺ phenotype by the calcium phosphate precipitation method (23) using the DNA plasmid, px-1 (24) containing the cloned HSV-1 TK gene. Cells were maintained in Glasgow-modified Eagle's medium supplemented with 10% calf serum and 10% tryptose phosphate broth.

Viruses—SC16, an HSV-1 strain (25), and drug-resistant mutants made from it were used in this study. The isolation of the acyclovir-resistant mutant SC16 S1 has been described (21). SC16 Tr7, another

[1] The abbreviations and trivial names used are: HSV, herpes simplex virus; HSV-1, HSV, type 1; acyclovir, 9-(2-hydroxyethoxymethyl)guanine; BVdU, (E)-5-(2-bromovinyl)-2'-deoxyuridine; acyclo-GTP, 9-(2-hydroxyethoxymethyl)guanine triphosphate; SDS, sodium dodecyl sulfate; S1, HSV-1 strain SC16 S1; Tr7, HSV-1 strain SC16 Tr7; B3, HSV-1 strain SC16 B3.

acyclovir-resistant virus, was isolated in BHK TK1 cells infected with SC16 in the presence of 4 μM acyclovir. SC16 B3, a BVdU-resistant mutant, was made by infecting BHK cells with SC16 in the presence of 30 μM BVdU (26). This mutant was kindly provided by Dr. H. J. Field, Department of Pathology, Cambridge University, United Kingdon.

Viruses were cloned and stocks were prepared in BHK cells using low multiplicity infections.

Measurement of Virus Sensitivity to Acyclovir and BVdU—Plaque reduction assays were performed using BHK monolayers as described previously (27).

Virus Infection of BU-BHK Cells for Enzyme Preparations—Confluent monolayers of BU-BHK cells (about 2×10^8) were infected at a multiplicity of 10 plaque-forming units/cell. Infection was allowed to proceed for 18 h, after which the cells were harvested into the medium, pelleted, and frozen at -70 °C until required.

Measurement of Thymidine Kinase Induced in Infected Cells—BU-BHK cells were infected and assayed for thymidine kinase activity as described previously (22).

Extraction and Purification of Virus DNA Polymerase and Thymidine Kinase—The purification procedure used was designed to permit purification of both HSV DNA polymerase and thymidine kinase from the same infected cell extract.

All buffers (except those used for thymidine kinase affinity column chromatography) contained, in addition to the indicated amount of potassium phosphate buffer, pH 7.5, 2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM thymidine, and 20% glycerol. All steps were performed at 4 °C. Infected cell pellets were thawed and resuspended in 4 volumes of extraction buffer containing 250 mM potassium phosphate buffer, pH 7.5. Following disruption by ultrasonic vibration, the sonicate was centrifuged at 15,000 rpm in a Sorvall RC-2B rotor for 30 min. The supernatant was passed through a DEAE-cellulose column, equilibrated with extraction buffer to remove nucleic acids. The flow through was collected and diluted to a concentration of 50 mM potassium phosphate buffer, pH 7.5 then applied to a second DEAE-cellulose column, equilibrated with 50 mM potassium phosphate buffer, pH 7.5. Thymidine kinase was recovered in the column flow through and HSV DNA polymerase activity was eluted with 250 mM potassium phosphate buffer, pH 7.5. HSV DNA polymerase was further purified through phospho- and DNA-cellulose columns as previously described (28).

Fractions containing thymidine kinase activity from the second DEAE-cellulose column were pooled and solid ammonium sulfate was added to 60% saturation. The precipitate formed was collected by centrifugation, dissolved in 3 ml of thymidine kinase affinity column buffer A (0.05 M Tris-HCl, pH 7.5, 2 mM dithiothreitol, and 10% glycerol) and applied to a thymidine-Sepharose affinity column equilibrated with buffer A. A sequence of elution buffers (all containing 2 mM dithiothreitol, 10% glycerol, increasing concentrations of Tris-HCl, pH 7.5, and thymidine) were applied to the affinity column and thymidine kinase was eluted with a linear gradient of thymidine (100—300 μM), Tris-HCl, pH 7.5 (0.2–0.3 M), and KCl (0–1.2 M). Peak thymidine kinase fractions were pooled, glycerol and bovine serum albumin were added as stabilizers to 30% and 0.5 mg/ml, respectively, and the fractions were stored at -20 °C. Enzyme preparations were desalted by Sephadex G-25 filtration prior to use.

Enzyme Assays—Thymidine kinase was assayed at 37 °C essentially as described by Klemperer *et al.* (29) using [^{14}C]thymidine as substrate at 30 μM (1.6 μCi/ml). However, the reaction mixture with purified thymidine kinase also contained 2 mM dithiothreitol and 0.5 mg/ml of bovine serum albumin, in addition to 5 mM ATP, 5 mM MgCl$_2$, and 0.02 M sodium phosphate buffer, pH 6. When thymidine was replaced by alternative substrates the following concentrations were used: [^{14}C]acyclovir, 500 μM (6.2 μCi/ml) and [^{3}H]BVdU, 42 μM (10 μCi/ml). A unit of thymidine kinase activity is defined as the amount of enzyme catalyzing the conversion of 1 pmol of substrate/min at 37 °C under the reaction conditions described.

DNA polymerase assays were performed at 37 °C essentially as described by Ostrander and Cheng (28). Standard HSV DNA polymerase reaction mixtures (0.1 ml) contained 50 mM Tris-HCl, pH 8.0, 200 mM KCl, 4 mM MgCl$_2$, 0.5 mM dithiothreitol, 0.2 mg/ml of bovine serum albumin, 12.5 μg of activated calf thymus DNA, made by the method of Baril *et al.* (30), 0.1 mM each of dATP, dGTP, and dCTP and 5 μM [^{3}H]TTP (2.5 μCi/ml). When K_i values for acyclo-GTP were determined, [^{3}H]TTP was replaced in the reaction mixture by [^{3}H] dGTP and cold TTP (at 0.1 mM) was added. A unit of DNA polymerase activity is defined as the amount of enzyme catalyzing the

incorporation of 1 pmol of TMP or dGMP/min at 37 °C under the reaction conditions described.

Radiolabeled substrates were obtained from the Radiochemical Centre, Amersham, United Kingdom, except [^{14}C]acyclovir and [^{3}H] BVdU which were gifts from Dr. G. B. Elion, Burroughs Wellcome Co., Research Triangle Park, NC, and Dr. E. De Clercq, Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium, respectively.

Antiviral Compounds—The following drugs were gifts: acyclovir from Dr. Elion, Burroughs Wellcome Co.; BVdU from Dr. De Clercq, Rega Institute; acyclo-GTP and BVdUTP were prepared according to the procedure of Ruth and Cheng (7).

SDS-Polyacrylamide Gel Electrophoresis—Protein samples were precipitated with 7% trichloroacetic acid overnight at 4 °C and resuspended in SDS gel sample buffer (0.1 M dithiothreitol, 2% SDS, 24 mM Tris-HCl, pH 6.8, 20% glycerol, 0.2% bromphenol blue). Samples were loaded onto a 3% polyacrylamide stacking gel and separated on a 15% polyacrylamide gel as described previously (22). Following electrophoresis the gels were fixed in 50% methanol, 12% acetic acid, washed in 10% ethanol, 5% acetic acid to remove excess SDS, and silver-stained by the method of Merril *et al.* (31).

Protein Concentration Estimations—Protein concentration was estimated by the method of Bradford (32).

RESULTS

Resistance to Antiviral Drugs—The sensitivities of the three drug-resistant mutants of HSV-1 to both acyclovir and BVdU are shown in Table I. All three mutants were resistant to the drug used in their isolation, showing ED$_{50}$ values at least 100-fold higher than those obtained with wild type virus. The acyclovir-resistant mutants, S1 and Tr7, also showed some cross-resistance to BVdU although the BVdU-resistant mutant, B3, showed no resistance to acyclovir (26). Also included in Table I are data showing the level of thymidine kinase induced by these viruses assessed following high multiplicity infection of BU-BHK cells. Tr7 and B3 induced similar levels to wild type enzyme but S1 induced approximately 30% (22).

Previous experiments on thymidine kinase induced by the mutant S1 using crude enzyme preparations had shown it to have many properties which distinguish it from the wild type enzyme. However, although the changed properties of thymidine kinase probably account for the acyclovir resistance of this mutant, we had not directly excluded the possibility of an additional change in DNA polymerase (21, 22). Similarly, our

TABLE I

Virus sensitivities to acyclovir and BVdU and thymidine kinase induction in infected cells

Virus	ED$_{50}$[a]		Thymidine kinase activity[b]
	Acyclovir	BVdU	
	μM		
SC16	0.10	0.03	100
S1	10	0.54	30
B3	0.05[c]	30[c]	110
Tr7	22	0.37	130

[a] The sensitivity of viruses to acyclovir and BVdU was determined by plaque reduction in BHK cells. Preformed cell monolayers were infected with 100–250 plaque-forming units of virus and different concentrations of inhibitor added. Plaques were allowed to develop for 2–3 days and the cells were then fixed and stained. A plot of the reduction in plaque number (relative to no drug control) against drug concentration was constructed and the dose required for 50% plaque reduction (ED$_{50}$) was obtained from the graph.

[b] Thymidine kinase activity induced in infected cells was determined as described previously (22). BU-BHK cells were infected with virus at high multiplicity and harvested after 18 h. Cells were resuspended at 3×10^7 cells/ml in 0.01 ml of Tris-HCl, pH 7.4, sonicated, and centrifuged at 100,000 $\times$ *g* for 45 min. The supernatant was assayed for thymidine kinase activity, which is expressed in the table as a percentage of that induced by SC16.
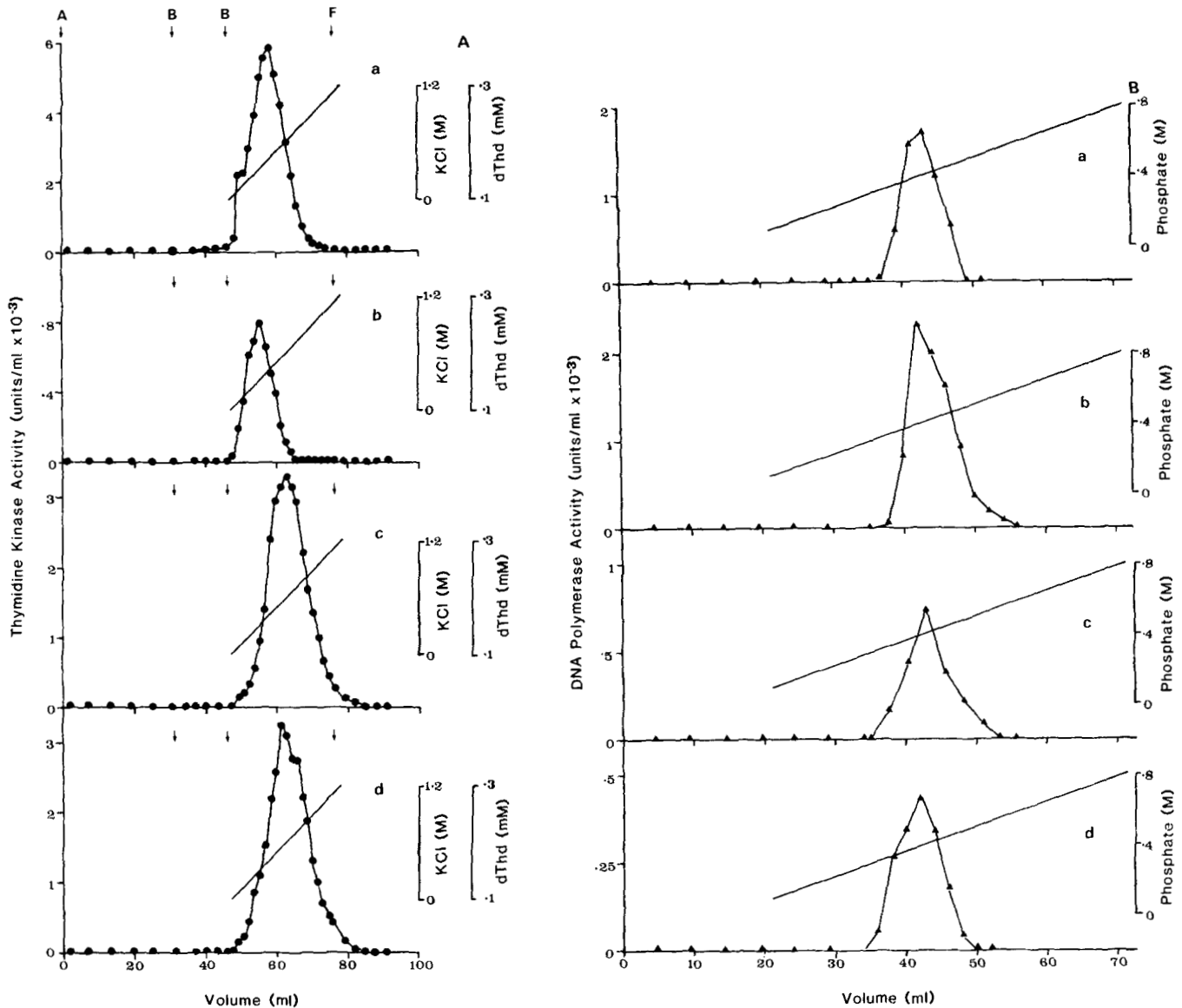
[c] Taken from Field and Neden (26).

FIG. 1. **Elution profiles of HSV DNA polymerases and thymidine kinases from affinity columns.** Infected cell extracts, prepared as described under "Materials and Methods," were passed through two DEAE-cellulose columns (equilibrated with 250 μM and 50 mM phosphate buffer, pH 7.5, respectively) and HSV DNA polymerase and thymidine kinase were further purified by affinity column chromatography. A, material eluted in the flow through from the second DEAE-cellulose column (with a phosphate buffer concentration of 50 μM) was pooled and ammonium sulfate was added to 60% saturation. The precipitate was resuspended in buffer A and applied to a thymidine-Sepharose affinity column (equilibrated with buffer A). The column was washed with increasing concentrations of Tris-HCl, pH 7.5, thymidine (*dThd*) and KCl (addition of buffers is indicated by *arrows*) and thymidine kinase was eluted with a gradient of buffers B and F, *i.e.* KCl (0–1.2 M), Tris-HCl, pH 7.5 (0.2–0.3 M), and thymidine (100–300 μM). All elution buffers contained 2 mM dithiothreitol and 10% glycerol; in addition, buffer B contained 0.2 M Tris-HCl, pH 7.5, and 100 μM thymidine and buffer F contained 0.3 M Tris-HCl, pH 7.5, 1.2 M KCl, and 300 μM thymidine. The amount of thymidine kinase activity loaded onto each column was: *a*, SC16 = 7.8 × 10⁴ units; *b*, S1 = 7.14 × 10³ units; *c*, B3 = 6.9 × 10⁴ units; and *d*, Tr7 = 2.4 × 10⁵ units. B, material eluted from the second DEAE-column with 250 mM phosphate buffer, pH 7.5, was pooled and dialyzed overnight to reduce the phosphate concentration to 100 mM. This was applied to a phosphocellulose column (equilibrated with 100 mM phosphate buffer, pH 7.5), DNA polymerase was eluted with a phosphate gradient (0.1–0.5 M), and the peak HSV DNA polymerase fractions were pooled and applied to a DNA cellulose column (equilibrated with 100 mM phosphate buffer, pH 7.5). DNA polymerase was eluted from this column with a phosphate gradient (0.1–0.8 M). All buffers contained, in addition to potassium phosphate buffer, pH 7.5; 2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20% glycerol. The amount of DNA polymerase activity loaded onto the DNA-cellulose column was: *a*, SC16 = 1.35 × 10⁴ units; *b*, S1 = 1.6 × 10⁴ units; *c*, B3 = 5.6 × 10³ units; *d*, Tr7 = 3.6 × 10³ units.

preliminary experiments with thymidine kinases induced by Tr7 and B3 had also indicated changes in those enzymes,[2] but we had not investigated the properties of the virus-specified

DNA polymerases and furthermore these experiments had also been carried out with crude enzyme preparations. We therefore purified both the thymidine kinase and DNA polymerase induced by each mutant and the wild type virus and investigated the properties of the purified enzymes.

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[2] B. A. Larder and G. Darby, unpublished results.

1st DEAE　2nd DEAE　Affinity
a b c d　a b c d　a b c d
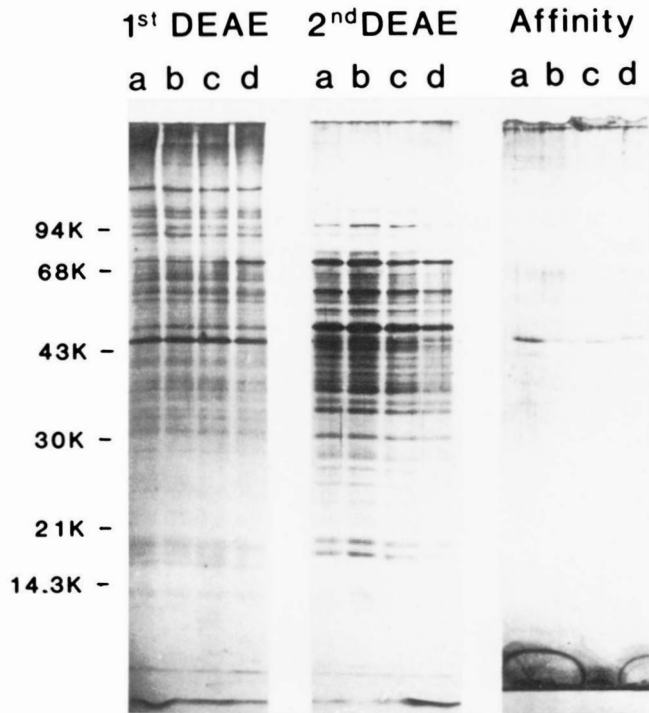

94K -
68K -
43K -
30K -
21K -
14.3K -

FIG. 2. **SDS-polyacrylamide gel electrophoresis of proteins at different stages of thymidine kinase purification.** Protein was precipitated from samples with 7% trichloroacetic acid at 4 °C overnight, resuspended in SDS gel sample buffer, and separated on a 15% polyacrylamide gel. Following electrophoresis, the proteins were fixed in the gel and silver-stained by the method of Merril *et al.* (31). *1st DEAE,* pooled flow through recovered from the first DEAE-cellulose column (2 μg of protein was loaded/tract), *2nd DEAE,* pooled flow through recovered from the second DEAE-cellulose column (1.5 μg of protein loaded/tract). *Affinity,* pooled peak thymidine kinase fractions from the thymidine affinity column (0.4 μg of protein loaded/tract). *a,* SC16; *b,* S1; C, B3; *d,* Tr7. It was estimated that the pooled material from the first DEAE-cellulose columns contained 49% of total protein, the pooled material from the second DEAE-cellulose columns 3.4%, and the pooled peak thymidine kinase fractions 0.1% of total protein.

Purification of Enzymes—The purification scheme was designed to allow us to isolate the virus-induced DNA polymerase and thymidine kinase from the same infected cell extract. It was a combination of protocols described previously for wild type enzymes and resulted in similar yields and degrees of purification to those reported earlier for each procedure used independently (28, 33). The final stage in the purification of each enzyme was affinity column chromatography. The elution profiles from these columns are shown in Fig. 1. The yields and specific activities of the mutant enzymes were similar to those obtained with wild type enzyme. It was noticeable that although the DNA polymerase eluted from the DNA cellulose columns at similar salt concentrations there were apparent differences in the elution profiles of the thymidine kinases from thymidine-Sepharose columns, with S1 eluting earlier, and B3 and Tr7 later than wild type enzyme.

We have previously shown that this purification scheme resulted in DNA polymerase preparations containing two specific major polypeptide species, revealed by silver staining following electrophoresis in SDS-polyacrylamide gels (34). A similar approach was used to examine the purified thymidine kinase preparations as the purity of thymidine kinase following affinity column chromatography has not been previously established. In each case a single major polypeptide species was observed (Fig. 2) with $M_r = \sim 45,000$. This was similar in size to the thymidine kinase subunit identified in extracts of ^{35}S-labeled HSV-1-infected cells (35–37).

Identification of Resistant Functions—As indicated above, studies with crude extracts of thymidine kinase induced by the mutant S1 had shown that this enzyme had a changed substrate specificity. This was revealed in kinetic studies using crude enzyme preparations in which it was shown that the enzyme had a higher K_m (thus, decreased affinity) for the natural substrate thymidine and a loss of ability to bind the analogue acyclovir (22). Similar approaches have been used to show that the DNA polymerase induced by acyclovir and phosphonoformic acid-resistant mutants have altered affinities for acyclo-GTP or phosphonoformic acid compared to wild type enzyme (34, 38). We therefore examined the properties of the enzymes induced by the three drug-resistant
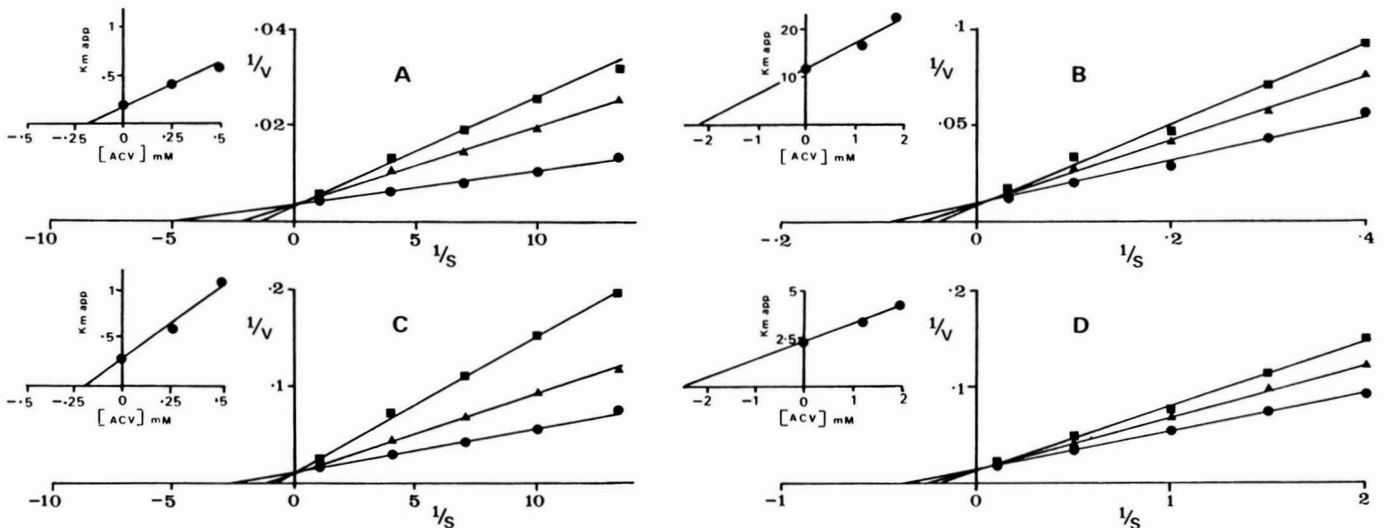

FIG. 3. **Inhibition of thymidine phosphorylation by acyclovir (ACV).** Reaction rates were determined at different concentrations of thymidine and two fixed concentrations of acyclovir. (ATP-Mg^{2+} concentration was kept constant at 5 mM.) Lineweaver-Burk plots are shown. Amount of acyclovir in the reaction mixture; A, SC16 thymidine kinase, no acyclovir (●), 0.25 mM acyclovir (▲), 0.5 mM acyclovir (■); B, S1 thymidine kinase, no acyclovir (●), 1.18 mM acyclovir (▲), 1.88 mM acyclovir (■); C, B3 thymidine kinase, no acyclovir (●), 0.25 mM acyclovir (▲), 0.5 mM acyclovir (■); D, Tr7 thymidine kinase, no acyclovir (●), 1.18 mM acyclovir (▲), 1.88 mM acyclovir (■). K_i values were evaluated from the replots of apparent K_m *versus* acyclovir concentration (showed in *insets*). S, concentration of thymidine (micromolar) in the reaction mixture; V, picomoles of thymidine phosphorylated/min/ml of enzyme.

viruses, looking particularly for a decrease in ability of thymidine kinases to bind either the natural substrate thymidine or its analogues, or impaired ability of DNA polymerases to recognize triphosphates or triphosphate analogues.

The affinities of the thymidine kinases for thymidine were assessed by measuring the Michaelis-Menten constants (K_m values) using thymidine as substrate and their affinities for acyclovir and BVdU by measuring the inhibition of thymidine phosphorylation by the appropriate analogues (Figs. 3 and 4). A similar approach was used to measure the K_m values for dGTP and TTP of the purified DNA polymerases and also the K_i values for acyclo-GTP and BVdU-triphosphate using dGTP and TTP as substrates, respectively. The data are summarized in Table II. Essentially the DNA polymerase purified from mutant and wild type infected cells showed no differences in K_m or K_i values: the values obtained were similar to those in earlier reports for HSV-1 DNA polymerase (5–7).

In contrast, significant differences were observed between the mutant and wild type thymidine kinases. The K_m values for thymidine of the thymidine kinases induced by the acyclovir-resistant mutants Tr7 and S1 (2.5 and 10 μM) were 10- and 50-fold higher, respectively, than that observed for the wild type enzyme. Similarly the K_i values of the mutant enzymes for acyclovir were increased by approximately 10-fold compared to wild type enzyme. The thymidine kinase induced by the BVdU-resistant mutant B3 showed little difference in K_m for thymidine (K_m 0.3 μM compared with K_m for thymidine for the wild type enzyme of 0.2 μM). However, the ability of BVdU to inhibit phosphorylation of thymidine by the mutant enzyme (K_i 4.5 μM) was considerably reduced (K_i for wild type enzyme 0.1 μM) suggesting that B3 thymidine kinase bound BVdU less strongly than SC16 thymidine kinase. Since the inhibition seen in each case was competitive, it is reasonable to assume that acyclovir and BVdU are alternative substrates
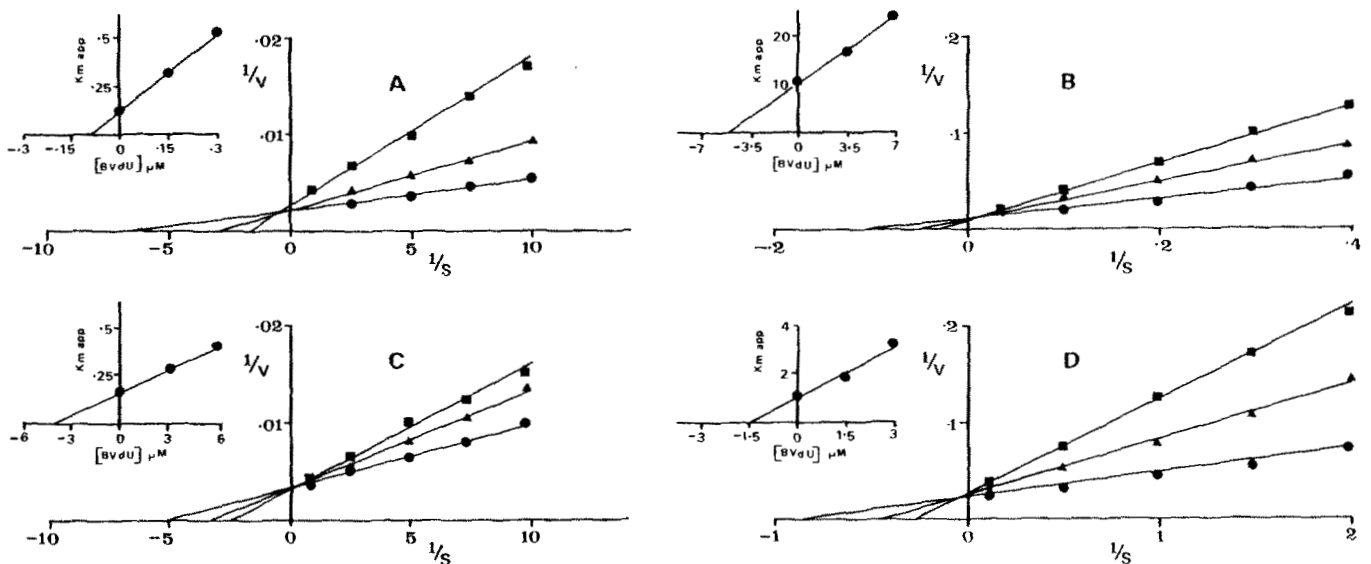

FIG. 4. **Inhibition of thymidine phosphorylation by BVdU.** Lineweaver-Burk plots are shown; these were constructed in a similar way to that described in the legend to Fig. 3, except that BVdU was used as the inhibitor instead of acyclovir. Amount of BVdU in the reaction mixture; *A*, SC16 thymidine kinase, no BVdU (●), 0.15 μM BVdU (▲), 0.3 μM BVdU (■); *B*, S1 thymidine kinase, no BVdU (●), 3.5 μM BVdU (▲), 7 μM BVdU (■); *C*, B3 thymidine kinase, no BVdU (●), 3 μM BVdU (▲), 6 μM BVdU (■); *D*, Tr7 thymidine kinase, no BVdU (●), 1.5 μM BVdU (▲), 3 μM BVdU (■). K_i values were evaluated from the replots shown in *insets* (apparent K_m *versus* BVdU concentrations). *S*, concentration of thymidine (micromolar) in the reaction mixture; *V*, a picomoles of thymidine phosphorylated/min/ml of enzyme.

TABLE II

Kinetic constants for DNA polymerase and thymidine kinase purified from wild type and drug-resistant HSV-infected cells

DNA polymerase and thymidine kinase were purified from either parental (SC16) or drug-resistant mutant virus infected cells as described under "Materials and Methods." Reaction velocities were measured at different substrate concentrations (TTP and dGTP for DNA polymerase and thymidine for thymidine kinase) and K_m values were evaluated from Lineweaver-Burk plots. K_i values were determined by measuring reaction velocities in the presence and absence of fixed concentrations of inhibitor. Inhibition of DNA polymerase by BVdU triphosphate was measured with limiting concentrations of [³H]TTP and saturating levels of dGTP, dATP, and dCTP. Inhibition by acyclo-GTP was measured using limiting concentrations of [³H]dGTP and saturating concentrations of TTP, dATP, and dCTP. All kinetic constants for thymidine kinase were determined at fixed, saturating levels of ATP-Mg^{2+} (5 mM).

	DNA polymerase					Thymidine kinase			
	SC16	S1	B3	Tr7		SC16	S1	B3	Tr7
	μM					*μM*			
K_m					K_m				
TTP	0.68	0.62	0.70	0.68	Thymidine	0.2	10	0.3	2.5
dGTP	0.67	0.67	0.67	0.67					
K_i					K_i				
BVdU-triphosphate	0.25	0.28	0.23	0.23	BVdU	0.1	5	4.5	1.5
Acyclo-GTP	0.015	0.017	0.013	0.013	ACV	200	2200	225	2500

TABLE III

Relative phosphorylation rates of thymidine, ACV, and BVdU by SC16 and mutant virus thymidine kinase

Substrate	Phosphorylation relative to thymidine[a]			
	SC16	S1	B3	Tr7
	%			
Thymidine	100	100	100	100
ACV	39	NP[b]	42	NP
BVdU	120	129	30	145

[a] Phosphorylation activity of thymidine kinase purified from SC16 and mutant virus-infected cells was measured using different radiolabeled substrates. The standard thymidine kinase assay reaction mixture (described under "Materials and Methods") containing either thymidine (30 μM), acyclovir (500 μM), or BVdU (42 μM) as substrate was used. Reaction velocities are expressed as a percentage of the thymidine phosphorylating rate for each enzyme.

[b] No phosphorylation measurable; levels therefore less than 1%.

for these enzymes and this was subsequently confirmed (see below).

Phosphorylation of Nucleoside Analogue Substrates—The purified thymidine kinases were directly tested for ability to phosphorylate radiolabeled acyclovir or BVdU as substrate, and this was compared in each case with the ability of the enzyme to phosphorylate thymidine. The results of these experiments are shown in Table III. As expected acyclovir was phosphorylated by SC16 thymidine kinase but to a lesser extent than thymidine or BVdU. At the concentration of acyclovir used in the assays (500 μM) no phosphorylation of acyclovir by S1 and Tr7 thymidine kinases was observed, which is consistent with earlier results using crude infected extracts (22).

All enzymes except B3 thymidine kinase phosphorylated BVdU with slightly greater efficiency than thymidine itself. The poor phosphorylation of this substrate by B3 thymidine kinase cannot be explained solely on the basis of the elevated K_i since the activity was determined at high substrate concentration (42 μM). There may also be a significant decrease in V_{max} for phosphorylation of BVdU although we have not attempted to measure this parameter directly since it is extremely difficult to measure absolute concentrations of enzymes.

DISCUSSION

The advent of effective and selective drugs for use against herpes infections (*e.g.* acyclovir and BVdU) has drawn attention to the potential problems posed by drug-resistant viruses. Although it is not yet clear how serious the threat may be, there are already reports that resistance is a problem confronting clinicians (39–41). In view of this we felt it worthwhile to investigate virus phenotypes which might exhibit resistance, particularly those with similar pathogenicity to the parent.

In this paper we have focused on three pathogenic TK$^+$ drug-resistant mutants derived from the same HSV-1 parental strain. Two had been derived by growth of virus in the presence of acyclovir (21) and the third by growth in BVdU (26). Initial studies indicated that all three mutants induced TK, B3 and Tr7 inducing levels similar to that of wild type enzyme and S1 inducing only about 30%. When tested for resistance to the drugs used in their selection, the mutants were all considerably less sensitive than their parental strain. Tr7 and S1 were somewhat resistant to BVdU although the results obtained with S1 were variable. Significantly, the BVdU-resistant mutant B3 showed no cross-resistance to acyclovir (26).

Previous work with S1 had shown it to be pathogenic in a mouse model system and the infection was not affected by

treatment with acyclovir (21). Similar results have been obtained with Tr7,[3] and B3 also appears to be pathogenic, infection with this latter mutant being refractory to treatment with BVdU but not acyclovir (26).

Thymidine kinase and DNA polymerase induced by each mutant were isolated and purified. Biochemical studies were then undertaken to investigate whether any of the enzymes induced had altered properties which might account for the resistance of the viruses to the drugs. Results with the DNA polymerase suggested that these enzymes were similar if not identical to the wild type enzyme. The K_m values measured using TTP as substrate for each enzyme were not significantly different nor were the values obtained using dGTP. Similarly the K_i values for acyclo-GTP were similar for each enzyme as were the K_i values for BVdU triphosphate. There was thus no evidence that any of the DNA polymerases induced by the drug-resistant variants differed from the wild type enzyme in their recognition of nucleoside triphosphates or their analogues.

In contrast to the results with DNA polymerases, the thymidine kinases induced by the mutants had properties which clearly distinguished them from the wild type enzyme. The K_m values for thymidine of S1 and Tr7 thymidine kinases were considerably higher, indicating lower affinities for the natural substrate. The weakened binding was also reflected in the K_i values for acyclovir. The K_i for acyclovir of the wild type enzyme was 200 μM, but both mutant enzymes had K_i values greater than 2 μM. In earlier studies with S1 thymidine kinase we obtained similar results for the thymidine K_m, but we had been unable to measure a K_i with acyclovir (22).

The thymidine kinase induced by the mutant B3 had a K_m for thymidine (0.3 μM) similar to that of the wild type enzyme (0.2 μM). However, the K_i for the analogue BVdU was considerably higher (4.5 μM) than that of the wild type enzyme (0.1 μM), again indicating a decrease in affinity of the mutant enzyme for the drug, but in this case not associated with decreased affinity for the natural substrate.

In experiments using acyclovir or BVdU as inhibitors of thymidine phosphorylation inhibition appeared competitive, confirming the likely site of action of these analogues to be the thymidine binding site and suggesting that they may act as alternative substrates for the enzyme. This was confirmed directly using the radiolabeled drugs as substrates (Table III). In these experiments it is probably only valid to compare the relative phosphorylating activities of each individual enzyme, comparisons between enzymes being considerably more difficult in the absence of precise quantitative data on the amount of each enzyme present in the assays. Thus, the wild type enzyme phosphorylated BVdU with greater efficiency than thymidine and acyclovir at rather lower efficiency. B3 thymidine kinase, in contrast, phosphorylated BVdU poorly, but acyclovir was phosphorylated in relative terms as well as by the wild type enzyme. The poor phosphorylation of BVdU may explain why B3 is significantly more resistant to BVdU in tissue culture than S1, even though the K_i values for BVdU of both S1 and B3 thymidine kinases are similarly elevated. Tr7 and S1 thymidine kinases were similar to wild type enzyme in as much as they phosphorylated BVdU more efficiently than thymidine. However, there appeared to be no detectable phosphorylation of acyclovir by these mutant thymidine kinases. This was not surprising in view of the reduced binding affinity of these enzymes for acyclovir (Fig. 3). These data suggest that there would be little phosphorylation of acyclovir in S1- and Tr7-infected cells compared with that in cells infected by the wild type virus.

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[3] H. J. Field and B. A. Larder, unpublished results.

It is clear from the results that all three mutant viruses express enzymes with impaired ability to phosphorylate the drugs used in their selection. It is difficult to know whether the differences are sufficient to account for the observed resistance of these viruses to the drugs, but in the absence of any apparent changes in DNA polymerase this would seem the most likely explanation.

Thus, in conclusion, we have shown that drug-resistant viruses may retain a TK$^+$ phenotype (thus retaining pathogenicity) but induce altered thymidine kinases which show lower affinity for nucleoside analogue substrates than the wild type enzyme. This confirms our earlier work with S1 but shows that this is not a unique phenomenon. Like S1, Tr7 is an acyclovir-resistant mutant, but its thymidine kinase has properties which clearly distinguish it from S1 thymidine kinase. In addition, this type of response is not restricted to acyclovir but may also be seen in mutants resistant to BVdU. We would suggest that this is a general strategy which may be adopted by HSV to confer resistance to those nucleoside analogues which require initial phosphorylation by HSV thymidine kinase, and that adoption of this strategy may not necessarily result in a significant decrease in pathogenicity.

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