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Inhibition of Herpes Simplex Virus Type 2 Replication by Thymidine

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Replication of herpes simplex virus type 2 (HSV-2) was impeded in KB cells which were blocked in their capacity to synthesize DNA by 2 mM thymidine (TdR). The degree of inhibition was dependent upon the concentration of TdR. In marked contrast, HSV-1 is able to replicate under these conditions. The failure of HSV-2 to replicate is probably due to the inhibition of viral DNA synthesis; there was a marked reduction in the rate of DNA synthesis as well as the total amount of HSV-2 DNA made in the presence of 2 mM TdR. We postulated that the effect of TdR on viral replication occurs at the level of ribonucleotide reductase in a manner similar to KB cells. However, unlike KB cells, an altered ribonucleotide reductase activity, highly resistant to thymidine triphosphate inhibition, was found in extracts of HSV-2-infected KB cells. This activity was present in HSV-2-infected cells incubated in the presence or absence of TdR. Ribonucleotide reductase activity in extracts of HSV-1-infected KB cells showed a similar resistance to thymidine triphosphate inhibition. These results suggest that the effect of TdR on HSV-2 replication occurs at a stage of DNA synthesis other than reduction of cytidine nucleotides to deoxycytidine nucleotides.

We reported recently that herpes simplex virus type 1 (HSV-1) replication is unimpeded in KB cells incubated in the presence of 2 mM thymidine (TdR) (3). This concentration of TdR blocks host cell DNA synthesis and subsequent cell division (1, 2, 3, 15, 17). These observations suggested that at least one aspect of DNA replication utilized by HSV differs significantly from that of the host cell. This difference was explained by the finding of a "new" viral-induced ribonucleotide reductase enzyme activity in the cell after infection and by the observation that this enzyme was markedly resistant to inhibition by thymidine triphosphate (TTP), a negative effector of ribonucleotide reductase (1, 3, 9, 15). The "new" enzyme relieved the TdR block to DNA synthesis by insuring a pool of deoxycytidylate precursors for replication of virus.

The oral form of HSV has been shown to differ significantly from the genital form (HSV-2) in a number of biological, biochemical, and biophysical characteristics (12). Therefore, we investigated the capacity of HSV-2 to replicate in KB cells in the presence of excess TdR.

We present evidence here to show that HSV-2 is unable to replicate in the presence of 2 mM TdR. The failure to replicate is probably due to the inhibition of viral DNA synthesis. However, a ribonucleotide reductase with properties similar to the enzyme found in HSV-1-infected cells was also present in HSV-2-infected cells. This suggests that the TdR block occurs at a stage of DNA replication other than reduction of cytidine nucleotides to deoxycytidine nucleotides.

MATERIALS AND METHODS

Cell cultures. Conditions for growth and maintenance of KB cells and baby hamster kidney (BHK) cells have been previously described (3, 4).

Virus strains and titrations. Three strains of HSV-1, HF, KOS, and CL, and three strains of HSV-2, Savage (SAV) HSV (381) and VF, were used. The KOS, HSV (381), and VF strains were kindly provided by L. H. Moss III, Ohio State College of Medicine. Strains VF and CL were recent isolates and were passed three times in BHK monolayers prior to use. Preparation of high-titered virus stock as well as assay by plaque titrations were performed as described previously (3).

Cell synchronization. KB cells were synchronized by the double TdR block method (3, 4). Briefly, KB cells were treated with 2 mM TdR for 18 to 20 h. The cells were washed free of TdR by centrifugation and resuspended in fresh warm medium to reverse the block, and then allowed to grow for an additional 9 to 12 h. At this time, 2 mM TdR was added for another

period of 18 to 20 h. Infections were started 1 h prior to reversal of the second-TdR block.

DNA synthesis in synchronized cell cultures. Pulse-labeling experiments were carried out as previously described (3, 4) employing 0.2 μCi of [^{3}H]hypoxanthine per ml (specific activity 5 to 10 Ci/mmol, New England Nuclear Corp.). Methods for the separation of DNA and RNA as well as the determination of radioactivity were previously described (3, 4). Radioactive-labeled DNA was obtained as previously described except that [^{3}H]hypoxanthine was employed as the isotope (3, 4). Separation of viral DNA from cellular DNA was carried out by CsCl density gradient centrifugation (3, 4).

Protein and RNA synthesis in synchronized cell cultures. Infected or uninfected KB cells (generally 2 × 10^5 cells/ml) were incubated at the times indicated with 0.2 μCi of [^{3}H]L-amino acid mixture per ml (New England Nuclear Corp.) to measure protein synthesis or [^{3}H]hypoxanthine (0.2 μCi/ml) to estimate RNA synthesis. Incorporation of label was stopped by pouring the cells onto iced phosphate buffered saline (PBS). The cells were collected by centrifugation and resuspended in 5 ml of cold 5% trichloroacetic acid. The resulting precipitate was washed three times with trichloroacetic acid and dissolved in 0.1 N NaOH. The samples were added to Aquasol and the radioactivity was measured.

Preparation of extracts for enzyme assay. The preparation of cell-free extracts has been previously reported (3). The extracts were stored at −100 C in a Union Carbide liquid nitrogen refrigerator. Under these storage conditions ribonucleotide reductase activity remains stable for several years.

Ribonucleotide reductase assay. The conversion of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP) was measured by a modification of the method described by Cohen (3) to include procedures in the assay described by Setlow (14). The standard reaction mixture (0.25 ml) contained: 10 mM Tris, pH 7.8, 2.5 mM ATP, 1.4 mM dithiothreitol (DTT); 2.8 mM $MgCl_2$; 0.02 mM CDP, 1.5 μCi of [^{3}H]CDP (specific activity 13 Ci/mmol; Schwartz-Mann) and enzyme extract. The samples were incubated at 30 C for 15 min, and the reaction was stopped by addition of 0.25 ml of 2 N HCl. The precipitated material was separated from the supernatant fluid by centrifugation. The supernatant fluid was heated at 100 C for 15 min, then evaporated to dryness in a desiccator containing solid KOH and P_2O_5. Samples were redissolved in 50 μliters of water and a 25-μliter portion was analyzed by paper chromatography (3). The rate of formation of dCMP in cell extracts prepared from uninfected KB cells and KB cells infected with HSV-1 or HSV-2 was linear for at least 25 min and was proportional to extract added in the range of 0.2 to 0.8 mg of protein. Each assay was run in duplicate and the results presented are the average of duplicate assays which varied by 10%. One unit of ribonucleotide reductase activity is defined as that amount of enzyme which catalyzes formation of dCMP at a rate of 1 nmol per mg per h. Protein concentration was determined by the method of Lowry et al. (8).

RESULTS

Replication of infectious virus in the presence and absence of 2 mM thymidine. Table 1 shows a typical experiment in which the number of PFU formed by three strains of HSV-1 in the TdR-blocked KB cells was nearly as high as the number formed in the control cells (no TdR). In contrast, TdR had a significant inhibitory affect on replication of the three genital strains. The titers were 200- to 400-fold lower in KB cells containing TdR than in controls (no TdR) and were similar to background levels found in 3 h-infected cultures. Furthermore, this decrease in titer in the presence of TdR was not due to release of HSV-2 into the supernatant fluid. The total amount of extracellular virus was at least one log lower than that of cell-associated virus. These results indicate that HSV-2 replication in synchronized KB cells was inhibited by 2 mM TdR. In the following experiments, one strain of HSV-2 (Savage) and one strain of HSV-1 (HF) was employed.

Dose response relationship of thymidine concentration to virus synthesis. Figure 1 shows the dose response relationship between the amount of HSV-2 synthesized by KB cells and the concentration of TdR present in the medium. In the range 2 μM to 2 mM, every 10-fold increase in TdR concentration produced approximately a one log decrease in virus titer. Complete inhibition occurred at 2 mM TdR and no inhibition was observed at 2 μM. These results indicate that HSV-2 replication was inhibited at a concentration of TdR which also inhibited cellular DNA synthesis and division in uninfected KB cells (3, 4) and that this

TABLE 1. *Effect of excess thymidine on the replication of HSV types 1 and 2*

HSV virus strain employed	PFU[a]	
	No thymidine (20 h)	Thymidine (20 h)
HSV-1		
HF	2.8×10^7	1.6×10^7
KOS	1.6×10^8	6.0×10^7
CL (wild)	6.8×10^7	2.0×10^7
HSV-2		
SAV	2.6×10^8	1.0×10^{4b}
HSV (381)	3.6×10^7	1.0×10^{5b}
VF (wild)	4.0×10^6	1.8×10^{4b}

[a] Each value represents the average of duplicate samples. See legend in Fig. 1 for procedure.

[b] Values similar to background of PFU found at 3 h postinfection.

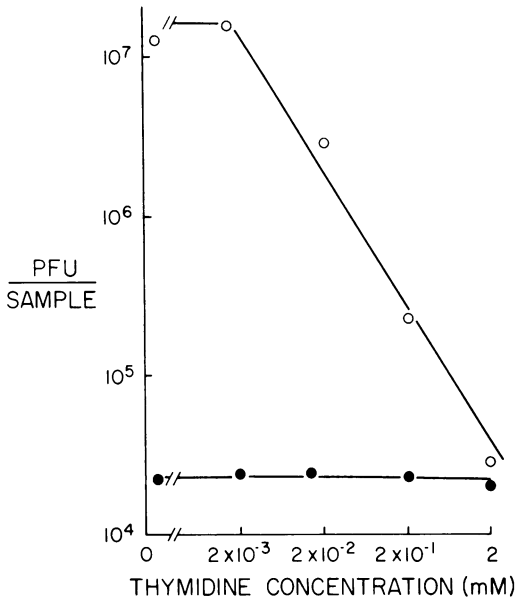

FIG. 1. *Dose response relationship of TdR concentrations to virus synthesis. Cultures were synchronized by the double TdR block method. The cells were washed to remove TdR and infected at an input multiplicity of 10 PFU/cell in the presence of varying concentrations of TdR as indicated in the figure. The virus was allowed to absorb for 1 h. Unabsorbed virus was eliminated by three washes with minimal essential medium containing the corresponding TdR concentration. Twenty-four hours later the cells were harvested and washed with PBS, and cell associated virus was determined. Symbols: O, PFU per sample with varying concentrations of TdR; ●, background virus in KB cells containing TdR harvested at 3 h postinfection.*

inhibition of new virus synthesis was dose dependent.

Rate of DNA synthesis in HSV-2-infected and uninfected KB cells in the presence and absence of excess TdR. Synchronized KB cells were infected with HSV-2 in the presence and absence of 2 mM TdR. The rate of DNA synthesis was measured by pulse labeling cell samples for 1 h with [^{3}H]hypoxanthine.

Figure 2A shows the rate of DNA synthesis in uninfected KB cells. When KB cells were washed free of TdR, a wave of DNA synthesis occurred. The rate of DNA synthesis increased rapidly for 3 to 4 h, then decreased by 8 h. The cell number remained constant for 8 to 10 h post reversal and then doubled. A second round of DNA synthesis (corresponding to the second S phase) commenced at 12 to 14 h. When the cells were incubated in the presence of TdR, the rate of host cell DNA synthesis remained constant at less than 8% of the reversed culture and there was no increase in cell number. The results are essentially the same as previously reported (3).

The pattern of DNA synthesis in synchronized cultures infected with HSV-2 was markedly different (Fig. 2B). In the absence of TdR, the rate of DNA synthesis reached a maximum approximately 5 h after infection and was never greater than 30% of that found for uninfected KB cells. DNA synthesis in the infected cells then declined slowly for at least 16 h.

In contrast, the rate of DNA synthesis in TdR-blocked cells increased only slightly by 3 h after infection and subsequently returned to a low level. These results show that DNA synthesis in TdR-blocked infected cells occurs at a much reduced rate and suggests that the inhibi-

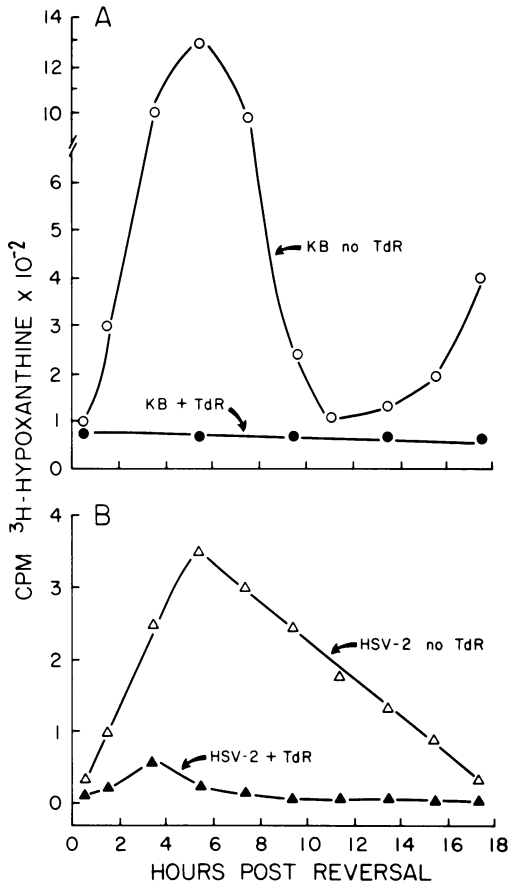

FIG. 2. *Rate of DNA synthesis in synchronized cultures of uninfected KB and HSV-2-infected cells in the presence and absence of TdR. The rates were determined by pulse labeling 5.0-ml cell samples (2×10^5 cells/ml) with [^{3}H]hypoxanthine for 1 h, as indicated in Materials and Methods. Symbols: panel A, O, uninfected cells; ●, uninfected in the presence of 2 mM; panel B, Δ, infected cells, and ▲, infected in the presence of 2 mM TdR cells were infected at an input multiplicity of 20 PFU/cell.*

tion of viral replication may be due to an inhibition of viral DNA synthesis.

Before conclusions could be drawn concerning HSV-2 production and the level of DNA synthesis, it was necessary to show that the radioactive DNA made after infection was viral and to determine how much viral DNA was made in the presence of TdR.

CsCl density gradient analysis of DNA synthesized in HSV-2-infected KB cells in the presence or absence of excess TdR. Synchronized KB cells infected with HSV-2 were incubated in the presence or absence of TdR. Tritium-labeled hypoxanthine was added to the cells 1 h after reversal and 9 h later they were harvested. The extracted DNA was analyzed by buoyant density ultracentrifugation in CsCl. The radioactive profiles obtained are shown in Fig. 3. Two bands of radioactivity were detected in infected cells incubated in the presence or absence of TdR. One band (1.700 g/cm^3) corresponds in buoyant density to KB DNA (3) and the second (1.725 g/cm^3) to HSV DNA (3). The amount of viral DNA synthesized in the presence of TdR was markedly reduced. From these studies and others we estimate that only 10% of the HSV-2 DNA found in the controls was synthesized in the presence of TdR. These results differ from those previously reported for HSV-1 (3). In that case excess TdR had little effect on the rate or amount of viral DNA synthesis.

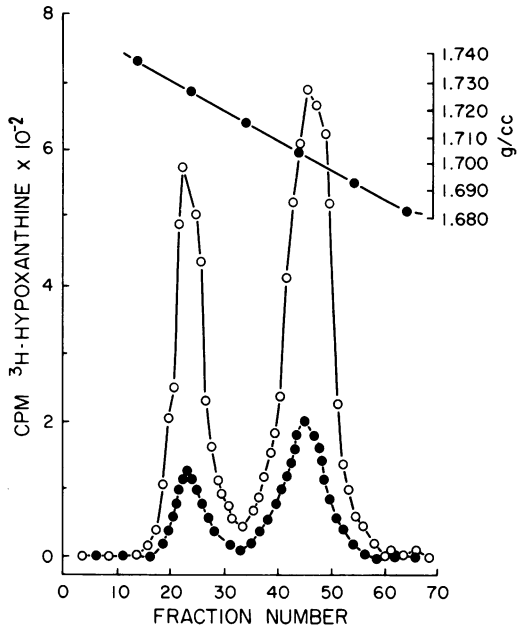

FIG. 3. *CsCl density gradient analysis of DNA synthesized in KB cells in the presence or absence of excess TdR. In each case, [^{3}H]hypoxanthine was added at a time corresponding to 1 h after TdR reversal. The cells were then incubated for an additional 9 h. Symbols: O, DNA (2.8 μg) isolated from HSV-2-infected KB cells which were incubated in the absence of excess TdR; ●, DNA (5.8 μg) isolated from HSV-2-infected KB cells and incubated in the presence of excess TdR.*

Viral inhibition of KB cell RNA and protein synthesis. The observation that new viral DNA was made in HSV-2-infected cells in the presence of TdR suggests that some viral functions were expressed. Supportive evidence was obtained by examining another characteristic of HSV infection.

We asked whether HSV-2 infection of TdR-blocked KB cells resulted in the inhibition of RNA and protein synthesis. Cellular RNA and protein synthesis are not markedly inhibited in mammalian cells by excess TdR (2, 16, 17). When cells are infected with HSV, inhibition of host macromolecular synthesis is dependent upon production of viral-induced protein(s) (6, 13). Therefore, inhibition of host RNA and protein synthesis would constitute indirect evidence that viral functions are expressed.

Table 2 shows the effect of 2 mM TdR on RNA and protein synthesis in uninfected and infected KB cells. When the cells were incubated in the presence of TdR, neither RNA nor protein synthesis were markedly inhibited. In contrast, both RNA and protein synthesis in HSV-2-infected KB cells were markedly inhibited, and the extent of this inhibition was enhanced by the presence of TdR.

Ribonucleotide reductase activity in HSV-infected cells grown in the presence and absence of excess TdR. HSV-1 replicates in TdR-blocked KB cells (3). This characteristic has been ascribed to the production of a "new" or altered ribonucleotide reductase activity which is not subject to allosteric regulation by TTP (3). DNA replication by this virus is not regulated in the same manner as host cell DNA

TABLE 2. *Effect of TdR on uninfected and infected KB cell RNA and protein synthesis*

KB cell	TdR + or −	% Inhibition	
		RNA[a]	Protein[a]
Uninfected	−	0	0
Uninfected	+	5	12
Infected	−	57	68
Infected	+	93	88

[a] [^{3}H]hypoxanthine or ^{3}H-labeled amino acids was added at a time corresponding to 1 h after TdR reversal. The cells were then incubated for an additional 9 h in the presence or absence of TdR.

synthesis. Thus it was possible that the failure of HSV-2 to replicate would be reflected by the absence of a "new" ribonucleotide reductase activity similar to that found with HSV-1. Studies were undertaken to compare the ribonucleotide reductase activity found in uninfected cells and in cells infected with HSV-1 and HSV-2.

Ribonucleotide reductase activity in synchronized KB cells is periodic, being highest in extracts prepared at or just prior to reversal of the TdR block (3). Enzyme activity in the washed cells remains at a maximum for 6 to 8 h post reversal, then decays rapidly during the course of the mitotic cycle (3). In contrast, if the TdR block is not reversed, the activity of the enzyme remains essentially unchanged.

Synchronized KB cells were infected with HSV-1 or HSV-2 and washed free of TdR 1 h later. Cells were harvested at 3.5 and 9 h postinfection corresponding to the S and G2-M phases of the cell cycle. Figure 4A shows that reductase activity of uninfected KB cells was, as previously shown (3), highly sensitive to inhibition by TTP. Furthermore, the percent inhibition by TTP was identical for enzymes prepared at the two times in the cell cycle.

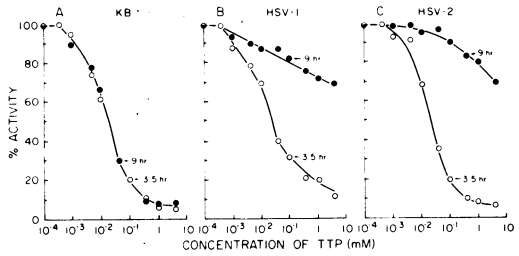

FIG. 4. *Effect of TTP on ribonucleotide reductase activity in extracts of uninfected and infected KB cells. A culture of KB cells was synchronized by the double TdR block method. One hour prior to reversal, one third of the culture was infected with HSV-1, one third with HSV-2, and the other third was mock-infected. Cells were infected at an input multiplicity of 25 PFU/cell, for both viruses. After 1 h of incubation the cultures were washed by centrifugation, resuspended in fresh medium, and allowed to grow. Samples were taken at 3.5 and 9 h after TdR reversal. The activities of each preparation were determined in the presence of varying concentrations of TTP. The standard assay conditions were used as described in Materials and Methods, except that TTP was added to samples as indicated in the figure. One hundred percent activity was determined by performing the assay in the absence of TTP. Symbols: ○, 3.5 h; ●, 9 h. Panel A, ribonucleotide reductase activity in extracts prepared from uninfected cells (3.5 h = 1.94 U, 9 h = 1.84 U); panel B, enzyme activity in extracts prepared from HSV-1-infected cells (3.5 h = 1.42 U, 9 h = 0.76 U); panel C, enzyme activity in extracts prepared from HSV-2-infected cells (3.5 h = 1.52 U, 9 h = 0.91 U).*

Figure 4B and C show that enzyme activity in extracts prepared at 3.5 h postinfection either with HSV-1 or HSV-2 had the same sensitivity to TTP as did the enzyme prepared from KB cells. However, by 9 h postinfection (Fig. 4B and C) ribonucleotide reductase activity in both HSV-1 and HSV-2 infected cell extracts was markedly resistant to inhibition. More than 70% of the maximum activity was present even in the presence of 4.0 mM TTP. These findings indicate that a "new" reductase activity is also present in extracts obtained from the HSV-2-infected cells. Moreover, the extent of TTP inhibition was the same for both viral-induced enzymes.

Even though HSV-2 induced a "new" ribonucleotide reductase, it was possible that the virus was unable to induce the enzyme in the presence of TdR. Therefore, KB cells were infected with HSV-2 in the presence of TdR. We observed that the presence of TdR during infection had no effect on the specific activity of the enzyme and no effect on the sensitivity of the enzyme to TTP. Moreover, the observation that dialysis had no effect on the reductase activity suggests that the insensitivity of the isolated HSV-2 enzyme to exogenous TTP was not due to an alteration in nucleotide pool size. No synergistic effect on ribonucleotide reductase activity was detectable when infected and uninfected extracts were mixed. The activity of a mixture of equal amounts of the two extracts was equivalent to the sum of the activity of each extract measured separately. From these studies, we conclude that the failure of HSV-2 to replicate in the presence of excess TdR cannot be explained by the inability of this virus to induce a "new" TTP-insensitive ribonucleotide reductase activity.

DISCUSSION

The present study compared the pattern of replication of HSV-1 and HSV-2 in KB cells growing in the presence or absence of excess TdR. Our findings suggest that TdR has different effects on the replication of HSV-1 and HSV-2. This observation adds an additional biochemical criterion which distinguishes HSV-1 from HSV-2 (12). Evidence has been presented that this difference is not due to a failure of HSV-2 to infect TdR-blocked KB cells. This evidence includes detection of new viral DNA albeit in low quantity, inhibition of host cell RNA and protein synthesis, and induction of an altered ribonucleotide reductase activity. Taken together, these results suggest that the failure of HSV-2 to replicate in TdR-blocked KB cells occurred at the level of viral DNA replication or later. Evidence that the

failure of HSV-2 to replicate in TdR-blocked cells is at the level of DNA replication was provided by the finding that both the rate of DNA synthesis as well as the total amount of viral DNA made were drastically reduced in the presence of excess TdR.

A problem raised by this study concerns the mechanism by which TdR inhibits HSV-2 replication. It would be reasonable that TdR affects viral replication at the level of ribonucleotide reductase as it does in the host cell (1, 2, 15). However, this explanation is not borne out by our results. Unlike the uninfected host cell, and similarly to HSV-1-infected cells, a "new" ribonucleotide reductase activity highly resistant to TTP inhibition is induced in HSV-2-infected cells regardless of the presence or absence of TdR.

Assuming that the ribonucleotide reductase is functional in HSV-2-infected cells in the presence of TdR, what function(s) is inhibited so as to prevent viral replication?

The TdR block may occur at a stage of DNA replication other than reduction of cytidine nucleotide to deoxycytidine nucleotide. Recent evidence indicates that the properties of some of the viral-induced enzymes found in HSV-1- and HSV-2-infected cell extracts differ in a number of characteristics including inhibition by TTP. Cooper (5) showed that TTP (0.1 mM) inhibited the phosphorylation of deoxycytidine by 85% in extracts of HSV-2-infected cells. However, TTP concentrations of 0.2 mM did not significantly inhibit deoxycytidine phosphorylation in extracts of uninfected or HSV-1-infected cells. Ogino et al. (10) showed a difference in the effect of TdR nucleotides on HSV-1- and HSV-2-induced TdR kinase. HSV-2 TdR kinase was much more sensitive to inhibition by TdR nucleotides than HSV-1 kinase.

Another possibility is that replication of HSV-2 may be dependent upon some phase of the host cell cycle. The replication of some viruses including equine abortion virus (7) and SV40 (11) is dependent upon some function(s) (reviewed in ref. 7 and 11) present only during the early S phase of the cell cycle. However, HSV-1 replication is independent of the KB cell cycle (3).

Experiments are now in progress to investigate the mode of action of TdR on HSV-2 replication.

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