Ribonucleotide Reductase Activity of Synchronized KB Cells Infected with Herpes Simplex Virus

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The replication of herpes simplex virus (HSV) is unimpeded in KB cells which have been blocked in their capacity to synthesize deoxyribonucleic acid (DNA) by high levels of thymidine (TdR). Studies showed that the presence of excess TdR did not prevent host or viral DNA replication in HSV-infected cells. In fact, more cellular DNA was synthesized in infected TdR-blocked cells than in uninfected TdR-blocked cells. This implies that the event which relieved the TdR block was not specific for viral DNA synthesis but allowed some cellular DNA synthesis to occur. These results suggested that HSV has a means to insure a pool of deoxycytidylate derivatives for DNA replication in the presence of excess TdR. We postulated that a viral-induced ribonucleotide reductase was present in the cell after infection which was not inhibited by thymidine triphosphate (TTP). Accordingly, comparable studies of the ribonucleotide reductase found in infected and uninfected KB cells were made. We established conditions that would permit the study of viral-induced enzymes in logarithmically growing KB cells. A twofold stimulation in reductase activity was observed by 3 hr after HSV-infection. Reductase activity in extracts taken from infected cells was less sensitive to inhibition by exogenous (TTP) than the enzyme activity present in uninfected cells. In fact, the enzyme extracted from infected cells functioned at 60% capacity even in the presence of 2 mM TTP. These results support the idea that a viral-induced ribonucleotide reductase is present after HSV infection of KB cells and that this enzyme is relatively insensitive to inhibition by exogenous TTP.

During recent investigations concerning the relationships between herpes simplex virus (HSV) replication and the mitotic cycle of KB cells (5), we found that a normal round of viral replication can occur when the concentration of thymidine (TdR) in the medium is sufficient to block host cell deoxyribonucleic acid (DNA) synthesis and cell division. This observation suggested that the pathway of DNA synthesis utilized by HSV differs somewhat from that of the host cells by virtue of the fact that it is refractory to high concentrations of TdR. Similar observations have been reported for pseudorabies virus (11), vaccinia virus (9), and *Chlamydia psittaci* (35).

The mechanism by which excess TdR inhibits mammalian DNA synthesis and subsequent cell division has been well studied (3, 7, 10, 25-27, 32, 37). A phosphorylated derivative, presumably thymidine triphosphate (TTP), acts as an allosteric inhibitor of the enzyme ribonucleotide reductase, which is responsible for the production of deoxycytidine nucleotides from cytidylate. When reductase is inhibited, the cells become

deficient in deoxycytidine triphosphate (dCTP), and neither DNA synthesis nor cell division can take place. In addition to the production of dCTP, ribonucleotide reductase is also involved in the production of all the other DNA nucleotide precursors (19, 21, 30).

We present evidence here to show that the unimpeded replication of HSV in the presence of excess TdR is due to a viral-induced ribonucleotide reductase. This enzyme differs significantly in some of its biochemical properties from the enzyme found in uninfected KB cells. More specifically, this reductase activity is refractory to inhibition by TTP at concentrations which inhibited host cell reductase activity. We have also established conditions which enabled us to demonstrate that ribonucleotide reductase activity is stimulated in synchronized KB cells as a consequence of HSV infection.

MATERIALS AND METHODS

Cell culture. Suspension cultures of KB cells were propagated in Eagle's minimal essential medium (MEM) modified for suspension culture and supple-

mented with 10% calf serum as previously described (5).

Virus preparation and infection. The procedures used for preparation of HSV (strain HF-oral form) pools and titration of the virus are the same as previously described (5). For infection we employed an input multiplicity of 200 plaque-forming units (PFU) of HSV per cell.

Cell synchronization. Suspension cultures of KB cells were synchronized by the double TdR-block method (3) as modified by Bello (1). In essence, KB cells growing at 36 C in suspension culture were treated with 2 mM TdR for 18 to 20 hr, centrifuged at $800 \times g$ for 10 min, resuspended in fresh warmed (36 C) medium to reverse the TdR block, and then allowed to grow for an additional 9 to 12 hr. At this time, 2 mM TdR was again added, and the cells were incubated for an additional 18 to 20 hr.

Rate of DNA synthesis in synchronized cell cultures. Pulse-labeling experiments were carried out by incubating 5.0 ml of infected or uninfected KB cells (generally 2.0×10^5 cells/ml) with 0.75 μ Ci of hypoxanthine-8- 14 C (specific activity 54.3 mCi/mmol; Schwartz BioResearch, Inc.) in a shaker bath at 36 C. Incorporation of label was stopped at the end of a 1-hr period by pouring the cells into iced phosphate-buffered saline. The cells were then collected by centrifugation at $800 \times g$ for 10 min and suspended in 5.0 ml of cold 5% trichloroacetic acid for 20 min. The resulting precipitate was washed three times with 5% trichloroacetic acid and dissolved in 0.1 N NaOH. DNA and ribonucleic acid (RNA) were separated as described by Bello (1). Samples were added to 10 ml of Aquasol (New England Nuclear Corp.) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Preparation of labeled DNA. Radioactively labeled cellular DNA was obtained from synchronized uninfected or infected KB cells by incubating the culture in medium containing 0.25 μ Ci of hypoxanthine-8- 14 C per ml for various periods of time as indicated. Labeled cells were pelleted, washed, and concentrated by centrifugation. Cellular or viral DNA, or both, were extracted from the pellet as previously described (5).

Cesium chloride density gradient centrifugation. Separation of viral DNA from cellular DNA was accomplished by CsCl density gradient centrifugation in a Spinco no. 40 fixed-angle rotor as described previously (5).

Preparation of extracts for enzyme assay. Synchronized KB cells were harvested, washed with 150 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.8, and concentrated by centrifugation. The cell pellet was suspended in 50 mM Tris containing 1 mM dithiothreitol (DTT) to a final concentration of 4×10^7 cells/ml. The resuspended cell pellet was exposed three times to 1-min periods of sonic treatment with 15-sec intervals in a 60W MSE sonifier (Measuring and Scientific Equipment, Ltd., London). Sonified cells were centrifuged in a Spinco model L2-50 centrifuge at $100,000 \times g$ for 1 hr, and the supernatant fluid was stored at -100 C. The ribonucleotide reductase and thymidine kinase activity of these cell-free extracts remained stable after storage for at least 6 months at this tem-

perature. Protein concentration was determined by the method of Lowry et al. (22) with crystalline bovine serum albumin as a standard.

Ribonucleotide reductase assay. The conversion of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP) was measured by a modification of the method described by Moore and Hurlbert (24). The standard reaction mixture (total volume 0.39 ml) contained 10 mM Tris (pH 7.8), 4.5 mM adenosine triphosphate (ATP), 3.5 mM $MgCl_2$, 3.5 mM DTT, 0.03 mM CDP, 1.5 μ Ci of 3H -CDP (13.3 Ci/mole; Schwartz BioResearch, Inc.), and enzyme extract. Paper chromatography (29) was employed to separate cytosine-containing compounds. Radioactivity was assayed in 10 ml of Spectrofluor (Amersham/Searle) scintillation fluid. The rate of formation of deoxycytidine phosphates in extracts prepared from either infected or uninfected cells was linear for 30 min. Reductase activity in uninfected cell extracts was proportional to protein concentration in the range from 0.2 to 1.6 mg of protein. In HSV-infected cell extracts, the activity was proportional to protein concentration between 0.2 and 1.0 mg of protein. Each assay was run in duplicate at two protein concentrations (usually 0.4 and 0.8 mg) to insure linearity. The reaction was carried out at 36 C for 25 min and was terminated by the addition of 1 ml of cold 1 M perchloric acid. One unit of ribonucleotide reductase activity is defined as that amount of enzyme which catalyzes deoxycytidine monophosphate (dCMP) synthesis at a rate of 1 nmole per 25 min.

Cytosine monophosphate and CDP served equally well as substrates for ribonucleotide reductase in both uninfected and infected cell extracts. Cytosine triphosphate was reduced at about one-half the rate of either the mono- or diphosphate form.

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.

Thymidine kinase assay. Thymidine kinase was assayed by using the method of Bello (*manuscript in preparation*) by measuring the rate of conversion of ^{14}C -thymidine to a form which was adsorbed by diethylaminoethyl (DEAE) cellulose. The assay conditions for thymidine kinase were as follows: the reaction mixture (final volume 0.25 ml) contained 3.5 mM $MgCl_2$, 80 mM Tris (pH 7.8), 6 mM ATP, 2 mM DTT, 0.1 mM TdR, 0.1 μ Ci of ^{14}C -thymidine (9.3 mCi/mole), and the cell extracts. The mixture was incubated at 36 C for 20 min and then placed in boiling water for 2 min to stop the reaction. Three milliliters of a DEAE cellulose suspension (1 g/60 ml) was added to the cooled tubes, mixed thoroughly, and centrifuged at $1,000 \times g$ for 5 min. The pellet was then washed three times with water and suspended in 2.0 ml of 1 N H_2SO_4 . The suspension was mixed and centrifuged, and 1 ml of the supernatant fluid was added to 10 ml of Aquasol prior to counting.

RESULTS

Rate of DNA synthesis in uninfected synchronized KB cells in the presence and absence of excess

TdR. A random culture of KB cells was synchronized by the double-TdR-block method. Eighteen hours after the second TdR treatment, half of the culture was infected with HSV (input multiplicity of 200 PFU/cell), and the other half was mock-infected with an extract prepared from normal KB cells. After 1 hr of incubation, these two cultures were each divided in half, and the four resulting cell suspensions were centrifuged. The pellets were suspended in fresh medium and treated as indicated in Fig. 1. Each culture was incubated for 20 hr at 36 C, and the rate of DNA synthesis was measured by pulse-labeling samples for 1 hr with ^{14}C -hypoxanthine.

When synchronized uninfected KB cells were resuspended in a medium lacking TdR, DNA synthesis (S phase) began shortly after reversal of the TdR block (Fig. 2). The rate of DNA synthesis in these cells increased rapidly for 4 to 6 hr, then decreased to approximately 10% of the maximum value by 10 hr. This low rate of cellular DNA synthesis remained constant until about 12 to 14 hr after removal of the TdR, when the second round of cellular DNA synthesis began. These results, which were obtained by using ^{14}C -hypoxanthine as the DNA precursor, are in accord with our earlier results obtained by using ^3H -TdR as precursor (5). In contrast, when uninfected (Fig. 2) KB cells were incubated in the presence of excess TdR, the rate of DNA synthesis was constant at less than 3% of the maximum value observed in the reversed KB culture.

Rate of DNA synthesis in infected synchronized KB cells in the presence and absence of excess TdR. Figure 3 illustrates the pattern of DNA synthesis that occurred when synchronized cultures were infected 1 hr prior to TdR reversal and then incubated in the presence or absence of excess TdR. In both cases, the rate of DNA synthesis increased rapidly and reached a max-

FIG. 2. Rate of DNA synthesis in a synchronized uninfected culture of KB cells in the presence (○) and absence (●) of excess thymidine. The rates were determined by pulse-labeling cells with ^{14}C -hypoxanthine for 1 hr and treating as described in the text.

imum approximately 4 hr after infection. The rate decreased to 50% of the maximum by 6 hr and continued to decrease until 12 hr after infection. These results suggest that the presence of excess TdR did not prevent DNA replication in HSV-infected KB cells. Kaplan (11) reported that excess TdR had no effect on the rate of DNA synthesis in pseudorabies-infected rabbit kidney (RK) cells.

CsCl density gradient analysis of DNA synthesized in synchronized KB cells in the presence

FIG. 1. Outline of basic experiment.

FIG. 3. Rate of DNA synthesis in synchronized cultures of KB cells infected with herpes simplex virus in the presence (Δ) and absence ($\blacktriangle$) of excess thymidine.

or absence of excess TdR. Figure 4A shows the results of density gradient CsCl centrifugation of DNA extracted and purified from uninfected KB cells released from the TdR block and cultured for 9 hr (1 to 10 hr after TdR reversal) in the presence of ^{14}C -hypoxanthine. One band of carbon label was detected corresponding in buoyant density to published values for KB cell DNA (1.700 g/cm^3) (5, 8).

Figure 4A also shows that KB cells incubated in the presence of excess TdR synthesized some new cellular DNA. Thus, the incorporation of labeled hypoxanthine into trichloroacetic acid-

precipitable material in the presence of excess TdR (Fig. 2) was due to low levels of cellular DNA synthesis. These results suggest that the inhibition of host cell DNA synthesis by 2 mM TdR was not complete. Studzinski and Lambert (34) reported that HeLa cells synthesized DNA at a low rate in the presence of excess TdR.

Figure 4B shows the results of CsCl centrifugation of DNA extracted from infected cells released from the TdR block and incubated for 9 hr in medium containing ^{14}C -hypoxanthine. Two bands of radioactive label were detected, one corresponding in buoyant density to KB DNA and the second to HSV DNA (21). In the absence of excess TdR, approximately 56% of the newly synthesized DNA was viral.

Figure 4C shows what happened when infected cells were labeled in the presence of excess TdR. In this instance, viral DNA represented 50% of the newly synthesized DNA. Thus, approximately the same proportion of viral DNA was labeled in the presence or absence of excess TdR. These data strengthen the idea that a normal round of viral DNA synthesis took place under conditions sufficient to inhibit cellular DNA synthesis in uninfected cells. Figure 4C also shows that significantly more cellular DNA was synthesized in infected TdR-blocked cells than in uninfected TdR-blocked cells. This suggests that the event which relieved the TdR block was not specific for viral DNA synthesis but allowed some cellular DNA synthesis to take place as well.

FIG. 4. CsCl density gradient analysis of DNA synthesized in synchronized KB cells in the presence or absence of excess thymidine (TdR). In each case, ^{14}C -hypoxanthine was added at a time corresponding to 1 hr after TdR reversal. The cells were then incubated for an additional 9 hr. (A) DNA isolated from uninfected KB cells washed free of TdR: 5,000 counts/min (2.5 μg) of ^{14}C -hypoxanthine-labeled DNA was added to the gradient ($\bullet\bullet$); DNA isolated from uninfected KB cells incubated for 9 hr in the presence of excess TdR: 600 counts/min (5.0 μg) of DNA was added to the gradient ($\circ$). (B) DNA isolated from KB cells infected 1 hr prior to the time of TdR reversal and incubated in the absence of excess TdR: 5,000 counts/min (2.5 μg) of DNA. (C) DNA isolated from KB cells infected 1 hr prior to the time of TdR reversal and incubated in the presence of TdR: 5,000 counts per min (3.8 μg) of DNA.

Replication of infectious virus in the presence of excess TdR. Table 1 shows that as much new infectious HSV was obtained from TdR-blocked KB cells as from untreated controls. In both cases, progeny virus could be detected by 6 hr after infection, and the maximum titer was reached by 12 hr. Apparently HSV replication was unaffected by a concentration of TdR sufficient to inhibit cellular DNA synthesis and cell division in uninfected cells.

Addition of equimolar amounts of deoxycytidine (CdR) to TdR-blocked mammalian cells has been shown to result in a resumption of DNA synthesis and cell division (18, 27, 37). It is possible that reversal of the TdR block after infection with HSV is not due to some viral function but is caused by the presence of CdR or one of its derivatives in the virus inoculum. We therefore designed experiments to test these possibilities.

Factors affecting DNA synthesis in TdR-blocked KB cells. A suspension culture of uninfected KB cells was synchronized (Fig. 1). One hour before the second TdR reversal, portions drawn from the culture were treated in the various ways shown in Table 2. The effect on DNA synthesis was followed over a 10-hr period by pulse-labeling the cells with ^{14}C -hypoxanthine. The rate of DNA synthesis 3 to 4 hr after infection was chosen for inclusion in Table 2 since this represented the time when the rate of DNA synthesis in infected cells was maximal.

When uninfected KB cells were incubated in the presence of equimolar amounts of TdR and CdR (treatment 1), more than 3,000 counts/min were incorporated into cellular DNA during the 1-hr pulse. This shows that CdR does indeed reverse the TdR block as has been previously reported (18, 27, 37). Cells which were mock-infected and incubated in the presence of TdR (treatment 2) incorporated less than 8% of this amount. This shows that the inoculum from un-

TABLE 2. *Factors affecting the rate of DNA synthesis in thymidine (TdR)-blocked KB cells*

Conditions of incubation of TdR-blocked KB cells ^a	Amt of ^{14}C label incorporated ^b (counts/min)
Deoxycytidine (2 mM).....	3,261
Mock-infected ^c	264
HSV inoculum (200 PFU ^d /cell).....	3,553
Centrifuged virus inoculum	
Supernatant fluid.....	290
Pellet (virus resuspended).....	2,956
Heat-inactivated virus inoculum ^e	326

^a Excess TdR (2 mM) was present in each case.

^b Amount of ^{14}C label incorporated into DNA per 2.5 ml of cells 3 to 4 hr after infection.

^c Mock-infected cells were treated with an extract of uninfected KB cells which was prepared in the same way as the virus inoculum.

^d Plaque-forming units.

^e Virus inoculum was heated at 100 C for 2 min.

infected KB cells did not contain enough CdR to reverse the TdR block. HSV-infected KB cells (treatment 3) synthesized DNA at approximately the same rate as observed in Fig. 3. Next, the virus inoculum (treatment 4) was partially purified by centrifugation at $100,000 \times g$ for 1 hr. This procedure removed more than 99% of the infectious virus from the supernatant fluid. Addition of this supernatant material to TdR-blocked cells (treatment 4A) had no effect upon incorporation of ^{14}C -hypoxanthine into DNA. Therefore, even if CdR derivatives are synthesized after HSV infection, they are not present in high enough concentration in the inoculum to reverse the TdR block. Addition of the resuspended virus (treatment 4B) resulted in restoration of DNA synthesis. Furthermore, heat-inactivated virus (treatment 5) lost the ability to induce DNA synthesis. These results show that reversal of the TdR block is associated with infectious HSV.

It appears that HSV has a mechanism to ensure a pool of CdR derivatives in the presence of excess TdR for DNA replication. The most plausible mechanism is that a modified ribonucleotide reductase is present in the cell after infection which is not inhibited by excess TdR. We therefore made a comparative study of the ribonucleotide reductase found in infected and uninfected KB cells.

Ribonucleotide reductase activity in uninfected synchronized KB cells in the presence and absence of excess TdR. Figure 5A illustrates that ribonucleotide reductase levels were quite high initially in extracts prepared from TdR-blocked KB cells. Enzyme activity in these cells remained at a maximal level for about 7 hr and then slowly

TABLE 1. *Effect of excess thymidine (TdR) on the replication of herpes simplex virus*

Treatment of infected cells	Time of harvest (hr postinfection)	PFU/sample ^a	
		Expt 1	Expt 2
TdR +	3 ^b	5.2×10^6	9.2×10^5
TdR -	3	4.4×10^6	10^6
TdR +	18	3.9×10^8	10^8
TdR -	18	3.2×10^8	9.3×10^7

^a Each value represents the average of duplicate samples. PFU, plaque-forming units.

^b Represents background value of virus that could not be removed by washing the cells.

FIG. 5. Ribonucleotide reductase activity in synchronized KB cells in the presence and absence of excess thymidine (TdR). A culture of KB cells was synchronized and divided as described in Fig. 1. At various times, samples were taken, and ribonucleotide reductase activity was determined as described in the text. (A) Reductase activity in uninfected KB cells incubated in the presence of excess TdR (○); reductase activity in uninfected KB cells incubated in the absence of TdR (●). (B) Reductase activity in herpes simplex virus (HSV)-infected KB cells (infected 1 hr prior to TdR reversal) incubated in the presence of excess TdR (△); reductase activity in HSV-infected KB cells incubated in the absence of TdR (▲).

decreased to about 77% of the maximum. This gradual decrease in activity might have been due to cell damage caused by prolonged exposure (27 hr) of the cells to excess TdR. In contrast, when the TdR block was reversed, the specific activity of the enzyme increased slightly for about 4 hr and then decayed rapidly during the course of the mitotic cycle. Enzyme activity was 12% of maximum by 12 hr after synchrony began, a time when DNA synthesis was at a minimum. Turner et al. (36) observed essentially the same periodicity of ribonucleotide reductase activity during the division cycle of L cells.

Ribonucleotide reductase activity in synchronized HSV-infected KB cells grown in the presence and absence of excess TdR. To support the proposal that an enzyme is viral-induced, it is desirable to show that enzyme activity increases as a consequence of viral infection. The *in vivo* studies of Kaplan and co-workers (2, 13, 14) were consistent with an increase in ribonucleotide reductase activity after infection of RK cells with pseudorabies. However, they were unable to detect ribonucleotide reductase activity in extracts of either infected or uninfected RK cells. Figure 5B shows that the extracts from HSV-infected synchronized KB cells initially had levels of reductase activity comparable to the levels found in uninfected cells (Fig. 5A). However, 3 hr after infection (Fig. 5B), enzyme activity decreases precipitously, reaching a constant level of approximately 40% of the maximum by 8 hr after infection. Essentially the same pattern of enzyme activity was found when synchronized cells were infected in the presence of TdR. In contrast to these results, reductase activity in uninfected synchronized cells (Fig. 5A) began to decay some 3 hr later in the division cycle. By 14 hr after reversal of the TdR block, reductase activity had decreased to approximately 12% of the maximum. By 14 hr after TdR reversal, ribonucleotide reductase activity (1.7 units/mg) was 4 times higher in infected cells than in uninfected cells (0.4 units/mg).

Stimulation of ribonucleotide reductase activity in synchronized HSV-infected KB cells. When cells are growing rapidly and host reductase activity is high, it is difficult to determine whether HSV infection stimulates enzyme activity. However, an approach was suggested based on the following observations: (i) synthesis of HSV is independent of G2-G1 phases of the cell cycle (5); (ii) during G2-G1, cellular (KB) DNA synthesis is minimal (15, 20); (iii) reductase activity decays rapidly (Fig. 5A) and approaches a minimum in G2-G1 phases. In view of these observations, we designed experiments to look for an unscheduled increase in reductase activity.

Cultures of synchronized KB cells were infected with 200 PFU of HSV per cell at 11 hr (G2-G1) after synchrony began (Fig. 6). Extracts taken from uninfected KB cells showed the same decay in reductase activity noted previously (Fig. 5A). The reductase activity decreased to a value of 0.4 units/mg by 14 hr after the start of synchrony. Extracts taken from the infected cells had the same amount of ribonucleotide reductase activity as extracts from uninfected cells for the first 2 hr after infection and then showed an increase (twofold) to 0.8 unit/mg by 3 hr after infection. This level remained constant for an additional 5 hr.

Stimulation of TdR kinase activity in HSV-infected synchronized KB cells. To determine whether the increase in reductase activity is related to viral replication, we studied TdR kinase under the same conditions. We chose this enzyme because it has been firmly established that infection of mammalian cells with HSV results in the synthesis of a new virus-specific TdR kinase activity (4, 17). Moreover, TdR kinase, like ribonucleotide reductase, is produced in a periodic manner in synchronized KB cells (Bello, *manuscript in preparation*). If synchronized KB cells were infected at a time when host TdR kinase

activity was low, a stimulation of this enzyme would be expected, as was noted for reductase activity. The conditions used for this experiment were identical to those used in Fig. 6.

We found that TdR kinase activity increased in uninfected cell extracts during the TdR block and for several hours after reversal and decreased markedly by 8 to 10 hr. Figure 7 shows that TdR kinase activity declined to a minimum by 16 hr and then began to increase between 18 and 21 hr. These results are in agreement with those reported by Bello (*manuscript in preparation*) for uninfected KB cells. During the first hour after infection with HSV (12 hr after TdR reversal), kinase activity decreased at the same rate in both infected cells and the uninfected controls. Subsequently, the activity of infected cells stabilized and then rose slightly and remained constant. By 5 hr after infection, the TdR kinase activity of the infected cells was approximately 1.5 times higher than the activity of uninfected controls. These results show that by infecting synchronized KB cells during G2-G1 we could study induction of enzymes in logarithmically growing KB cells.

Effect of TTP on ribonucleotide reductase. If a viral-induced ribonucleotide reductase is responsible for preventing the TdR block to viral DNA synthesis, then this activity should be

FIG. 6. Stimulation of ribonucleotide reductase activity in synchronized KB cells infected during G2-G1 phases of the cell cycle. Reductase activity in uninfected KB cells (○); reductase activity in herpes simplex virus-infected KB cells (△).

FIG. 7. Stimulation of thymidine kinase activity in synchronized KB cells infected during G2-G1 phases of the cell cycle. Enzyme activity in uninfected KB cells (○); activity in herpes simplex virus-infected KB cells (△). The conditions employed were identical to those in Fig. 6.

refractory, both in vivo and in vitro, to a TTP concentration which inhibits the host cell enzyme. A change in the regulatory properties of the enzyme after infection would also serve as evidence that the enzyme molecules remaining after infection are different from those present before infection.

Figure 8 shows the effect of varying concentrations of TTP on reductase activity in extracts of uninfected and infected KB cells. The host enzyme activity was inhibited by 50% in the presence of 0.05 mM TTP. The maximum inhibition of activity was 80% at 0.5 mM TTP. On the other hand, enzyme activity in infected cell extracts was

FIG. 8. Effect of thymidine triphosphate (TTP) on ribonucleotide reductase activity in extracts of uninfected and infected KB cells. A culture of KB cells was synchronized as described in the text. Eighteen hours after the second thymidine (TdR) treatment, half of the culture was infected with herpes simplex virus (HSV) (input multiplicity of 200 PFU/per cell), and the other half was mock-infected. After 1 hr of incubation, these two cell cultures were washed by centrifugation, resuspended in fresh medium, and distributed as indicated in Fig. 1. The activities of each preparation were determined in the presence of varying concentrations of TTP. Samples were taken at 5 hr after TdR reversal for the preparation of uninfected cell extract (specific activity = 3.4 units/mg of protein); infected cell extract, specific activity = 3.0 units/mg of protein. One hundred per cent activity was determined by performing the assay in the absence of added TTP. Ribonucleotide reductase activity in extracts prepared from uninfected cells (○); enzyme activity in extracts prepared from HSV-infected cells (△).

not inhibited at a TTP concentration of 0.05 mM. Furthermore, inhibition of enzyme activity by TTP was never greater than approximately 40% in extracts taken from infected cells.

These findings indicate that reductase activity in extracts taken from infected cells was less sensitive to inhibition by exogenous TTP than the enzyme present in uninfected KB cells. We conclude that the modified ribonucleotide reductase was capable of supplying CdR derivatives for a full yield of infectious virus in KB cells blocked by 2 mM TdR.

It is of interest that the uninfected cell extract still had residual reductase activity even in the presence of 2 mM TTP. This finding suggests that the host enzyme is still partially functional in vivo under these conditions (20% of capacity) and may explain the observation that a small amount of cellular DNA was synthesized in TdR-blocked cells (Fig. 4A).

Appearance of TTP-resistant ribonucleotide reductase activity in HSV-infected KB cells. If the stimulation of ribonucleotide reductase activity observed in Fig. 6 reflects the induction of an altered enzyme, this activity should also be refractory to inhibition by TTP.

Using the same extracts employed for the experiment in Fig. 6, we measured reductase activity in the presence of 0.05 mM TTP (Fig. 9). We previously determined that this concentration of TTP inhibited the host enzyme activity by 50%. For the first 2 hr after infection, enzyme activity was inhibited by 50% in the presence of 0.05 mM TTP. However, by 3 hr after infection, this concentration of TTP failed to inhibit the enzyme. In fact, the activity of the viral extracts was 3 times higher than the activity of uninfected extracts in the presence of 0.05 mM TTP.

These results support the idea that a viral-induced ribonucleotide reductase is present after infection of KB cells with HSV and that this enzyme is relatively insensitive to inhibition by exogenous TTP.

DISCUSSION

The results of the present study demonstrate that HSV replication is unaffected by the presence of 2 mM TdR, a concentration which essentially blocks host KB cell DNA synthesis. Mammalian ribonucleotide reductase, the enzyme responsible for production of CdR nucleotides as well as other DNA nucleotide precursors, is inhibited by a phosphorylated derivative of TdR, TTP (3, 7, 10, 25-27, 32, 37). Thus, addition of high concentrations of TdR to uninfected KB cells causes a shortage of DNA precursors. Nevertheless, a normal pattern of DNA synthesis, as well as the

FIG. 9. Effect of 0.05 mM thymidine triphosphate (TTP) on the stimulation of ribonucleotide reductase activity in synchronized KB cells during G2-G1 phases of the cell cycle. The conditions were identical to those in Fig. 6 except that each extract was assayed for enzyme activity in the presence of 0.05 mM TTP. Reductase activity in extracts prepared from uninfected KB cell; assay mixture contained no TTP (—) or 0.05 mM TTP (○). Reductase activity in extracts prepared from herpes simplex virus-infected cells; assay mixture contained 0.05 mM TTP (△). (See Fig. 6 for reductase activity in extracts prepared from infected cells with no TTP added to assay mixture.)

formation of new infectious virus, took place after HSV infection of KB cells in the presence of 2 mM TdR.

These results suggested that after infection ribonucleotide reductase was not inhibited by TTP and allowed the replication of viral DNA and progeny virus. We found that the enzyme from infected cell extracts was much less sensitive to inhibition by TTP than the host cell enzyme. In fact, the viral enzyme functioned at 60% capacity even in the presence of 2 mM TTP. This offers an explanation as to how enough CdR derivatives were produced by HSV in the presence of TdR to insure a full yield of infectious virus.

Having established that an altered ribonucleotide reductase activity is present after infection, we tested the possibility that this activity was produced as a consequence of infection. Evidence to support this idea is provided by our observation that reductase activity is higher in HSV-infected cell extracts than in uninfected extracts taken at times during G2-G1 phase in the cell

cycle. When synchronized KB cells were infected with HSV 1 hr prior to TdR reversal, the periodic pattern of reductase activity was drastically altered. Ribonucleotide reductase activity was as much as 4 times higher in infected cells than in uninfected cells. These results suggested the possibility that a "new" HSV-induced enzyme was present in infected cells. However, there are other possible explanations for this observation. For example, periodic decay of ribonucleotide reductase in uninfected synchronized cells may be due to proteolytic degradation of the enzyme molecule. It is possible that a product is synthesized during infection which alters the host enzyme and makes it less susceptible to degradation. Alternatively, HSV infection could inhibit the synthesis of a proteolytic enzyme which normally reduces the level of reductase in KB cells after S phase. It is unlikely that the higher levels of reductase activity in infected cells were due to continued synthesis of the enzyme, since little detectable protein synthesis was observed 10 hr after infection of synchronized KB cells (unpublished data).

Because these experiments were inconclusive, we designed a unique system that would enable us to determine whether ribonucleotide reductase activity is stimulated as a consequence of viral infection. We infected cells during G2-G1 (a time when host DNA synthesis was at a minimum) and looked for an unscheduled increase in reductase activity, which we expected would be evident when viewed against minimum host enzyme activity. Under these conditions there was a twofold stimulation in the reductase activity of infected cells by 3 hr after infection. [We previously found that HSV DNA synthesis was initiated during this time (5).] Furthermore, this enhanced reductase activity was not inhibited by 0.05 mM TTP, a concentration sufficient to inhibit the host enzyme by 50%.

Were the conditions reasonable to show that the stimulated reductase activity was a consequence of HSV infection? To answer this question we assayed TdR kinase activity under the same conditions. We chose this enzyme since it has been established that infection of mammalian cells with HSV results in the synthesis of a new virus-specific TdR kinase (4, 17). Moreover, TdR kinase, like ribonucleotide reductase, is produced in a periodic manner in synchronized KB cells (Bello, *manuscript in preparation*). When we infected KB cells with HSV during G2-G1, we detected a 1.5-fold stimulation of TdR kinase activity. These data show that we have been able to select conditions for studying the induction of enzymes in logarithmically growing KB cells.

It seems reasonable to conclude that infection of KB cells by HSV is accompanied by the induction of ribonucleotide reductase that is less sensitive to TTP than the uninfected host cell enzyme. Although we have no evidence that the post-infection enzyme is directly coded for by the viral genome (12, 15, 16, 33), these studies do offer an explanation of how HSV can replicate in the presence of concentrations of TdR sufficient to block host cell DNA synthesis.

Ribonucleotide reductase is not the only enzyme sensitive to allosteric control by TTP in mammalian cells. In addition to ribonucleotide reductase (31), TTP also inhibits CdR deaminase (23), TdR kinase (17), and CdR kinase (28). In each instance, when each of these enzymes was prepared from HSV-infected cells, it was markedly less sensitive to inhibition by TTP than its counterpart from uninfected cells (6, 17, 28). Induction of a new set of enzymes by HSV may free the virus from normal cellular controls and allow pyrimidine synthesis to continue in cells that have shut down their machinery for synthesizing DNA.

The ability of HSV to induce a number of enzymes required for DNA replication may explain, at least in part, why replication of HSV is independent of the G2-M-G1 phase of the cell cycle (5). In contrast, Lawrence (20) showed that the replication of equine abortion virus (EAV) DNA could be initiated only during the S phase of the cell cycle. We can postulate that the enzymes required for DNA replication, such as ribonucleotide reductase, must be present at a critical concentration for DNA synthesis to be initiated. If an infecting virus such as EAV could not induce the synthesis of these enzymes, replication of viral DNA would be entirely dependent upon existing cellular levels. However, the level of these enzymes decays rapidly at the end of the S phase. Thus, if infection took place during periods of the cell cycle other than the S phase, viral replication would be delayed until the next periodic rise in enzyme activity, corresponding to the next S phase.

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