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# Ribonucleotide Reductase Activity in Hydroxyurea-Resistant Herpesvirus Replication (39815)

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*Introduction.* Recently, we demonstrated that DNA synthesis and infectious virus replication of equine herpesvirus type 1 (EHV-1) are resistant to hydroxyurea (HU) at concentrations that rapidly and completely inhibit cellular DNA synthesis (1). Epstein-Barr virus, a herpesvirus, also replicates in the presence of this inhibitor (2, 3). Most studies indicate that HU blocks DNA synthesis by inhibiting the enzyme ribonucleotide diphosphate reductase and thereby reducing the intracellular pools of deoxyribonucleotides [see (1)]. Studies in our laboratory have eliminated certain mechanisms for the HU resistance of EHV-1 and indicate that protein synthesis is required for the drug resistance (1). Thus, the most likely mechanisms that would explain HU resistance involve alteration of ribonucleotide reductase to a state refractory to HU through modification of the host enzyme or synthesis of a viral-specified enzyme. In this report, we show that ribonucleotide reductase activity of EHV-1-infected cells has characteristics, including resistance to HU, different from those of the enzyme of control cells.

*Materials and methods. Cell culture and virus propagation.* L-M cells (mouse fibroblasts) were cultured routinely in YeLP medium (1, 4-6), and EHV-1, Kentucky A strain, was propagated and quantitated in L-M cells (5, 7). Hydroxyurea (Sigma Chemical Company, St. Louis, Mo.), when employed during infection, was added to a final concentration of 2.5 m*M*.

*Preparation of cellular extracts.* Mock-infected and EHV-1-infected L-M cells (multiplicity of infection = 10 to 20 PFU/cell) were incubated in sonication buffer [0.01 *M* Tris-Cl, pH 7.0, 0.65 m*M* dithiothreitol (DTT)] for 10 min in an ice bath, and cellular extracts were prepared by sonication (1-2 min; 10 KC Raytheon Sonic Oscillator, Raytheon Manufacturing Corp., Waltham, Mass.). This preparation was clarified by centrifugation at 15,000*g* for 15 min and stored at −70°. Protein was determined by the method of Lowry *et al.* (8). Partial purification of the enzyme was achieved by extensive dialysis followed by repeated acid precipitation and differential centrifugation, according to the method of Moore (9).

Column chromatography using Sephadex G-25 (Pharmacia, Piscataway, N.J.), hydrated in sonication buffer, was employed to remove salts as well as low-molecular-weight compounds (ATP, CTP, etc.) from the enzyme preparation. This method removed greater than 99% of all ATP as determined by reconstruction experiments with radiolabeled ATP (1 μCi/ml; 25 Ci/mmole).

*Enzyme assay.* Ribonucleotide reductase was assayed by the method of Moore (9). The 0.12-ml reaction mixture consisted of 8.3 m*M* potassium phosphate (pH 7.0), 4.4 m*M* ATP, 2.7 m*M* Mg acetate, 8.3 m*M* NaF, 0.06 m*M* $FeCl_3$, 6.2 m*M* dithioerythritol (DTE; Calbiochem, La Jolla, Calif.), and the substrate, CTP, labeled in the α-phosphate with $^{32}P$ (New England Nuclear, Boston, Mass.); in assays employing partially purified enzyme, [$^{32}P$]CDP was used as substrate. Reaction mixtures were incubated at 38° for 30 min. Preliminary assays demonstrated that the reaction was proportional to protein concentration in the range utilized (usually 1 mg) and that the reaction was linear through the first 30 min (data not shown).

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The reaction was stopped by the addition of 1 ml of 1 *M* perchloric acid and the precipitated protein was removed by centrifugation. Free nucleotides were hydrolyzed to the monophosphate form by heating to 100° for 10 min. Samples were cooled to 4°, neutralized with 4 *M* KOH, and incubated for 15 min in ice. After removal of the precipitated potassium perchlorate by low-speed centrifugation, the supernatant was assayed for deoxycytidine monophosphate (dCMP) by paper and thin-layer chromatography. Separation on paper (Whatman No. 1) was accomplished by ascending chromatography in a solution of isopropyl alcohol:HCl (12 *N*):$H_2O$ (65:18.4:16.6); 0.1 *M* dCMP was added as a marker. Areas corresponding to dCMP, located by radiochromatic scanning (Vanguard Instrument Corp., La Grange, Ill.) and uv absorption, were cut out and counted using Aquasol (New England Nuclear, Boston, Mass.) in a Beckman LS-230 scintillation counter. The second method involved chromatography on polyethyleneimine-cellulose (PEI-cellulose) (10). Aliquots (5 or 10 $\mu$l) were spotted onto 20 × 20-cm plastic sheets of PEI-cellulose (Schleicher and Schuell, Keene, N.H.) and developed up to 16 cm in a solution of 6 g of $Na_2B_4O_7 \cdot 10H_2O$, 3 g of $H_3BO_3$, and 25 ml of ethylene glycol in 70 ml of water. Spots were located by uv absorption. Areas corresponding to dCMP were removed and counted using Aquasol.

*Results. Ribonucleotide reductase activities of mock-infected and EHV-1-infected cells.* Ribonucleotide reductase activities of mock-infected and EHV-1-infected L-M cells were readily detected in complete reaction mixtures, and a 28% increase above the mock-infected activity was observed in the infected (6 hr PI) sonicate (Table I). Furthermore, deletion assays revealed the lack of an ATP requirement for *in vitro* activity of the infected cell preparation, whereas mock-infected extracts exhibited an absolute requirement for ATP. The ATP-independent activity of EHV-1-infected extracts increased from 30% to greater than 100% above that observed for infected extracts assayed in the presence of ATP. Although control and infected activities had similar requirements for DTE and NaF, the infected cell extract retained greater activity in the absence of iron.

*Reaction kinetics of CDP reductase activities.* As shown in Fig. 1, the CDP reductase activity of infected cells had significantly greater activity at lower substrate concentration than did that of the mock-infected cellular extracts. Analysis of the enzyme kinetics by the Lineweaver-Burk plot (insert, Fig. 1) revealed that a lower apparent $K_m$ for CDP was exhibited by ribonucleotide reductase in the EHV-1-infected cell extract. The apparent $K_m$ for the CDP reductase of infected cells was $4.2 \times 10^{-5}$ *M*, while that of the mock-infected cells was $9.1 \times 10^{-5}$ *M*.

*Inhibition of in vitro ribonucleotide reductase activity by hydroxyurea.* Studies comparing the sensitivity of the mock-infected and EHV-1-infected enzyme activities to HU (Fig. 2) revealed that the enzyme present in extracts of infected cells had decreased sensitivity to HU. The ribonucleotide reductase of the infected cells retained greater than 80% of its activity at HU con-

TABLE I. REQUIREMENTS FOR RIBONUCLEOTIDE REDUCTASE ACTIVITY IN MOCK-INFECTED AND EHV-1-INFECTED L-M CELLS.

| | Activity [$\alpha$-$^{32}$P] dCMP/mg of protein | | | |
|---|---|---|---|---|
| | Mock-infected | | Infected | |
| Reaction mixture | Picomoles | Percentage complete[a] | Picomoles | Percentage complete[a] |
| Complete[b] | 342.8 | 100 | 439.8 | 100 |
| −ATP | 0 | 0 | 588.3 | 133.8 |
| $-Mg^{2+}$ | 332.8 | 97.1 | 459.5 | 104.4 |
| $-Fe^{3+}$ | 172.0 | 50.2 | 324.1 | 73.7 |
| −DTE | 181.9 | 53.1 | 253.5 | 57.6 |
| −NaF | 174.4 | 50.9 | 274.3 | 62.4 |

[a] Percentage activity of the complete reaction.
[b] Complete reaction mixture as described in *Materials and methods*.

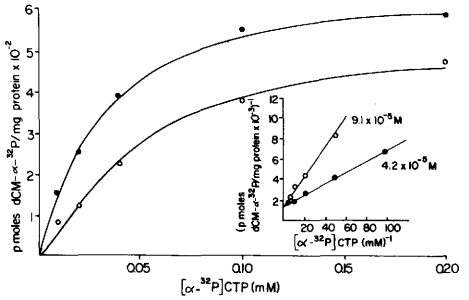


FIG. 1. Rate velocity of ribonucleotide reductase activity in EHV-1-infected and mock-infected L-M cell extracts. Samples prepared at 6 hr PI were assayed for 30 min at 38° with various substrate (CTP) concentrations as described in *Materials and methods*. Activity is expressed as the total picomoles of dCMP produced per milligram of protein for the 30-min reaction. Insert is the Lineweaver-Burk treatment of the data for evaluation of the $K_m$ of each enzyme preparation. Mock-infected cell extract (O——O); EHV-1-infected cell extract (●——●).

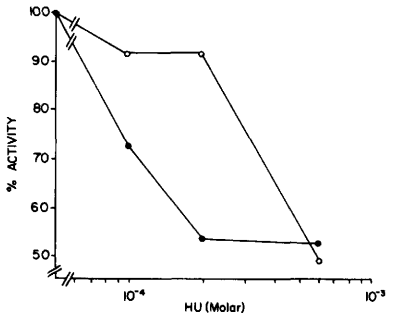


FIG. 2. HU inhibition of mock-infected and EHV-1-infected cell ribonucleotide reductase. Extracts prepared from mock-infected and EHV-1-infected cells (6 hr PI) were assayed in the presence of increasing concentrations of HU by the techniques described in *Materials and methods*. Enzyme activity at each concentration of HU is expressed as the percentage of that observed upon assay in the absence of inhibitor. Mock-infected cell extract (●——●); EHV-1-infected cell extract (O——O).

centrations below $2 \times 10^{-4}$ *M*. Thus, ribonucleotide reductase activity of infected cells has decreased sensitivity to inhibition by HU, although at high inhibitor concentrations significant levels of inhibition occurred.

*Time of synthesis of viral-induced CDP reductase activity in EHV-1-infected cells.* Previously, we reported that addition of cycloheximide prior to 4 hr PI completely prevented viral DNA synthesis in the presence of HU (1). However, if protein synthesis were inhibited at 4 hr PI or later, HU-resistant viral DNA synthesis continued at an elevated rate for several hours thereafter. It was shown that the viral-induced DNA polymerase was not responsible for this increase in DNA synthesis. An alternate explanation for this rise in viral DNA synthesis is that a protein(s) required for HU resistance became active between 3 and 4 hr PI.

To resolve whether or not the altered ribonucleotide reductase could be the protein that is implicated in HU-resistant DNA synthesis, experiments were undertaken to determine the time of appearance of the altered enzyme activity. Sonicates prepared at various times from EHV-1-infected and mock-infected cells were assayed under conditions that favor the detection of the altered enzyme activity: low substrate concentration, presence of $10^{-4}$ *M* HU, and deletion of ATP. The results are shown in Fig. 3. Mock-infected (0-hr-PI) extracts were found to have little CDP reductase activity (<5 pmole) at low substrate concentration, and this activity was inhibited approximately 25% by $10^{-4}$ *M* HU. No activity was detected in these extracts in the absence of ATP. Extracts prepared from EHV-1-infected cells at 2 hr PI were observed to have lower activity (<2 pmole) than that found for mock-infected cultures. The level of HU inhibition remained at 25%, and no activity occurred in the absence of ATP. By 3 hr PI, the activity detectable in the infected cell

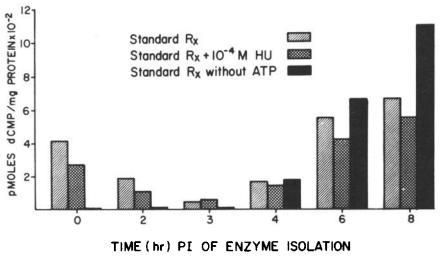


FIG. 3. Time of appearance of an altered ribonucleotide reductase activity in EHV-1 infection. Mock-infected (0-hr-PI) and EHV-1-infected preparations were assayed utilizing each of three separate reaction conditions: (i) standard reaction with no HU; (ii) standard reaction with $10^{-4}$ *M* HU; (iii) standard reaction without ATP. Reactions were performed as described in *Materials and methods*, with a substrate concentration of 0.02 m*M*.

extracts had decreased to a point approaching the lower limits of our assay. However, beginning at 4 hr PI, total activity at low substrate concentration began to increase. This activity was inhibited less than 20% by $10^{-4}$ M HU, and increased activity was observed in the absence of ATP. Similar results were observed in assays utilizing 6- and 8-hr-PI infected cell extracts, such that by 8 hr PI there was a 12-fold increase in ribonucleotide reductase activity in the ATP-independent reaction. In addition, HU added prior to infection had no effect on the appearance of the altered ribonucleotide reductase activity.

These results demonstrate that during EHV-1 infection of L-M cells there is an apparent inhibition of cellular ribonucleotide reductase early in the infection. In addition, an altered CDP reductase activity appeared between 3 and 4 hr PI, since the infected cell extracts prepared at 4 hr PI or later had decreased sensitivity to HU and did not require ATP, while activity of either mock-infected or early-infected (prior to 4 hr PI) extracts did not have these characteristics. Furthermore the correlation of these data with those previously reported (1) suggests that the altered ribonucleotide reductase activity is responsible for HU resistance.

*Discussion.* Of concern in this report is the mechanism by which EHV-1 replication is resistant to hydroxyurea. The enzyme ribonucleotide reductase, which is responsible for the production of deoxyribonucleotides, is thought to be inhibited by HU. Therefore, the virus must induce an alternate pathway for the synthesis of precursors that are necessary for the replication of the viral genome (1, 3). Reports on the presence of altered ribonucleotide reductase in mammalian cell lines (11, 12) and in herpes simplex virus-infected cells (13, 14) indicate that it is possible to alter the sensitivity of DNA synthesis (both host and viral) to feedback and metabolic inhibitors through the induction of an altered ribonucleotide reductase. Our results are in agreement with these findings and indicate that there are significant alterations in this enzyme activity in EHV-1-infected cells. The CDP reductase activity which appeared between 3 and 4 hour PI was shown to have no requirement for ATP, a lower apparent $K_m$, and decreased sensitivity to HU; the addition of HU prior to infection had no effect on the appearance of the altered activity.

Since highly purified enzyme preparations were not employed in these studies, it is not possible at the present time to ascertain whether EHV-1 codes for a viral-specified ribonucleotide reductase activity. However, the experiments employing partially purified ribonucleotide reductase confirmed that this enzyme activity is altered during EHV-1 infection, and studies attempting to purify this enzyme by utilizing substrate affinity column chromatography are in progress.

Feedback inhibition by deoxyribonucleotides appears to be an important regulatory device for control of ribonucleotide reductase (15). Of interest is the recent observation that deoxyribonucleotide triphosphate pool sizes are changed significantly during herpesvirus infection. It is apparent that ribonucleotide reductase activity of EHV-1-infected cells can function despite the presence of HU, the absence of ATP, and reduced levels of substrate concentration. Whether a viral-specified enzyme is present during EHV-1 infection or whether the host enzyme is modified so that its activity is resistant to changes in the available nucleotide pools remains to be elucidated.

*Summary.* The role of an altered ribonucleotide diphosphate reductase activity in the resistance of equine herpesvirus type 1 to hydroxyurea was demonstrated. A 28% increase in total CDP reductase was observed by 6 hr PI, and the enzyme activity of EHV-1-infected cells differed from that of mock-infected cells in several properties. Infected cell activity (i) did not require ATP for *in vitro* activity, (ii) exhibited a lower apparent $K_m$, and (iii) demonstrated a decreased sensitivity to HU added directly to the reaction mixtures. The altered CDP reductase activity was first detected at 3–4 hr PI, the time of initiation of viral DNA synthesis.

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