Biochemical Pharmacology, Vol. 34, No. 6, pp. 777–780, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

CAN RIBONUCLEOTIDE REDUCTASE BE CONSIDERED AS AN EFFECTIVE TARGET FOR DEVELOPING ANTIHERPES SIMPLEX VIRUS TYPE II (HSV-2) COMPOUNDS?*

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(*Received* 5 *April* 1984; *accepted* 19 *June* 1984)

Abstract—Herpes simplex viruses are known to induce virus specified ribonucleotide reductase (RR) in infected cells. RR is considered as a possible target for the development of antiviral agents. In this study, the role of RR in virus replication has been investigated. The sensitivity of RR to hydroxyurea (HU) from virus infected and uninfected HeLa S3 cells was similar with IC_{50} values of 0.12 and 0.14 mM. In the presence of 2 mM HU, and 10 μM tetrahydrouridine (THU), a cytidine deaminase inhibitor, the incorporation of [^{14}C]cytidine into viral DNA was found to be inhibited by 95%; [^{32}P]-incorporation into viral DNA under the same conditions was inhibited by 75%. The pool size of dCTP and dGTP was 50 and 70%, respectively, with no significant effect on dATP and dTTP pools in virus infected cells treated with 2 mM HU, as compared with virus infected cells receiving no drug treatment. HU at 2 mM could not inhibit HSV-2 yield by more than one log. These results suggest that virus RR is not an effective target for developing anti HSV-2 compounds.

Herpes simplex viruses are DNA viruses which are capable of inducing several virus specified enzymes in virus infected cells. These include DNA polymerase [1], deoxyribonuclease [1], thymidine kinase [2], ribonucleotide reductase [3], dUTPase and DNA uracil *N*-glycosylase [4]. The properties of these virus specified enzymes are quite different from those of their counterparts in human cells (for a review, see Ref. 5). Thus, approaches in developing selective antiviral compounds based on the presence of these virus specified enzymes in virus infected cells were taken by many laboratories, and useful antiviral compounds have been developed.

To design a strategy for developing antiviral compounds based on unique virus specified enzymes, it is important to understand the role of such enzymes in virus infected cells. This report describes our studies using hydroxyurea as a probe to examine the importance of ribonucleotide reductase activity in herpes simplex virus infected cells.

EXPERIMENTAL PROCEDURES

Materials. Hydroxyurea was purchased from the Sigma Chemical Co., St. Louis, MO. [2-^{14}C]CDP (476 mCi/mmole) was obtained from the New England Nuclear Corp., Boston, MA. [2-^{14}C]Cyd (43 mCi/mmole), [8-^{3}H]dATP (7 Ci/mmole), [*methyl*-^{3}H]dTTP (19 Ci/mmole) and [^{32}P]phosphoric acid were purchased from ICN Chemical and Radioisotopes Division, Irvine, CA. DNA polymerase (*Escherichia coli*) was purchased from Boehringer Mannheim, Indianapolis, IN. Tetrahydrouridine (THU) was a gift from Dr. R. Capizzi, Bowman Gray Medical School, NC. All other chemicals were reagent grade or better. A monoclonal antiserum (Bg 7) to HSV-2 ribonucleotide reductase was provided by Dr. S. Baccheti, McMaster University, Hamilton, Ontario. Materials used for cell culture were obtained from the Grand Island Biological Co., Grand Island, NY. HeLa S3 cells, cultured in monolayer or suspension, were maintained in RPMI 1640 supplemented with 5% dialyzed fetal calf serum and Kanamycin (100 μg/ml) unless otherwise specified. Cultures were determined to be mycoplasma free using the 4,6-diamidino-2-phenylindole fluorescence technique [6]. HSV-2 (333 strain) was used in all virus experiments. The antiviral activity of HU was determined in virus yield reduction assays as described previously [7], except that HeLa S3 and Vero cells were used in the present study.

Preparation of partially purified reductase. HeLa S3 cells in log phase were infected with HSV-2 at a multiplicity of infection (m.o.i.) of 5 plaque forming units (PFU) per cell, or were mock infected. Mock or virus infected cells ($1–2 \times 10^7$ cells) were harvested at 8 hr post virus infection by scraping into medium, and pelleted by centrifugation. The cell pellets were washed twice in phosphate-buffered saline (PBS; 0.14 M NaCl, 4.0 mM KCl, 0.5 mM Na_2HPO_4, 0.15 mM KH_2PO_4), and homogenized in 2.5 vol. of buffer [50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 7.5, 1 mM $MgCl_2$, 2 mM dithiothreitol] using a Dounce homogenizer. The homogenate was centrifuged at 10,000 *g* for 30 min, and the supernatant fraction was adjusted to 1% streptomycin sulfate. Precipitated material was removed by centrifugation. The supernatant fraction was adjusted to 45% ammonium

* Supported by Grant CH29 from the American Cancer Society.

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sulfate saturation and stirred for 30 min at 0–4°. After centrifugation (10,000 *g*, 30 min) the pellet was dissolved in homogenization buffer and dialyzed against the same buffer for 9 hr. The dialysate was used for measuring CDP reductase activity.

CDP reductase assay. CDP reductase was measured using Dowex-1-borate ion-exchange chromatography [8] as previously described [9], except that 60 μM $FeCl_3$ was included in the 30 min assay.

Incorporation of [^{32}P]phosphate and [^{14}C]Cyd into cellular and viral DNA. HeLa S3 cells were plated in a 6-well dish at 10^6 cells/10 cm^2 well. Once the cells were attached to the dish, the growth medium was removed and replaced with Joklik-Modified (low phosphate) medium containing 1% dialyzed fetal calf serum. Cells were then infected at 3 PFU/cell or mock infected, and incubated at 37° for a 1-hr adsorption period. The medium was then removed, and the cell layer was washed with PBS and replaced with 1 ml of medium containing various concentrations of HU (0 hr post virus infection). After a 2-hr incubation period, the medium was adjusted to a final THU concentration of 10 μM, and either [^{32}P]phosphate (25 μCi) or [2-^{14}C]Cyd (0.3 μCi; 43 μCi/μmole) was added to each well. At 8 hr post virus infection the samples were harvested by scraping the cells into the medium and centrifuging at 2000 rpm for 10 min. The cell pellets were washed twice in cold PBS and lysed overnight in buffer (10 mM Tris–HCl, pH 7.9, 1 mM EDTA, 100 mM NaCl, 0.5% sarkosyl and 1% sodium dodecyl sulfate) containing proteinase K at a final concentration of 1 mg/ml. This was followed by sodium iodide isopycnic gradient centrifugation and determination of alkali resistant radioactivity as previously described [10]. The alkali treatment as employed will remove all RNA contamination.

Deoxynucleoside triphosphate pools. HeLa S3 cells cultured in spinner flasks were infected at an m.o.i. of 5 PFU/cell. After a 1-hr adsorption period (0 hr post infection), cells were pelleted and resuspended in medium with or without 2 mM HU. At 0, 4 and 8 hr post virus infection neutralized perchloric acid extracts from 10^7 cells were prepared [11], and deoxynucleoside triphosphate (dNTP) pool measurements were done as previously described [12].

RESULTS

Effect of hydroxyurea on ribonucleotide reductase from virus infected cells. The presence of virally induced ribonucleotide reductase at 8 hr post virus infection was verified using a monoclonal antibody (Bg7) to the HSV-2 enzyme. The reductase activity in partially purified preparations from virus infected cells was precipitated by Bg7 antiserum, a highly specific preparation against HSV-2 ribonucleotide reductase, whereas reductase activity from mock infected cells could not be precipitated by the antibody (not shown). When the virus induced and cellular reductases were examined in terms of their sensitivity to HU, they exhibited a similar dose response with IC_{50} values of 0.12 and 0.14 mM respectively (Fig. 1). CDP reductase activity in the presence of 2 mM HU was not detectable in mock or virus infected reductase preparations. The reaction was

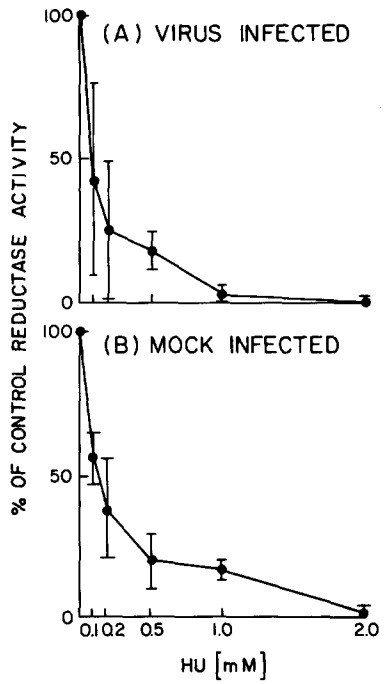

Fig. 1. Effect of HU on ribonucleotide reductase. The effect of HU was evaluated on enzyme isolated from HSV-2-infected (A) and mock-infected (B) HeLa S3 cells. For details, see Experimental Procedures.

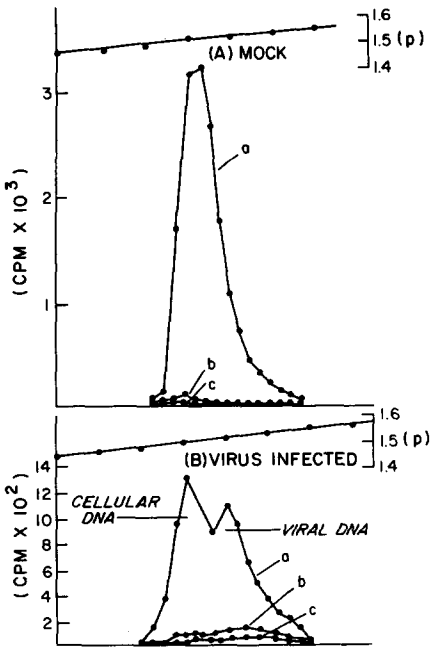

Fig. 2. [^{14}C]Cyd incorporation into DNA. Isopycnic centrifugation of DNA from mock (A) and HSV-2 infected (B) HeLa S3 cells, in the absence (a) or the presence of 1 mM HU (b) and 2 mM HU (c). (p) denotes gradient density in g/cm^3. For details see Experimental Procedures.

linear during the period of incubation. No significant elevation in total ribonucleotide reductase activity in virus infected cells as compared to mock infected HeLa S3 cells was observed.

Effect of hydroxyurea on the incorporation of [^{14}C] Cyd and [^{32}P]phosphate into cellular and viral DNA. The incorporation of [^{14}C]Cyd and [^{32}P]phosphate into the DNA of HSV-2 infected (5 PFU/cell) and mock infected HeLa S3 cells in the presence of 0, 1 and 2 mM HU was examined. The incorporation of [^{14}C]Cyd into DNA in the presence of THU is mainly dependent on the intracellular activity of ribonucleotide reductase whereas that of [^{32}P]-phosphate is not dependent on this activity. The conversion of [^{14}C]Cyd into intracellular ribonucleotides was not affected by HU, wheras the incorporation of [^{14}C]Cyd into mock and viral DNA was inhibited in a concentration-dependent manner by an average of 98 and 95%, respectively, in the presence of 2 mM HU (Fig. 2). In contrast, the incorporation of [^{32}P]phosphate into the DNA of mock and virus infected cells was inhibited by 98 and 75% as depicted in Fig. 3. Thus, greater than 20% of the viral DNA synthesis as measured by [^{32}P]-phosphate incorporation in comparison to infected cells in the absence of drug was ongoing in the presence of 2 mM HU which could inhibit intracellular reductase activity.

Effect of HU on dNTP pools. To examine the contribution of ribonucleotide reductase-mediated (*de novo*) dNTP formation to overall dNTP levels in the virus infected cell, dNTP pools were measured at 0, 4 and 8 hr post virus infection in the presence and absence of 2 mM HU. As shown in Fig. 4, all dNTP pools expanded after virus infection. The dATP and dTTP pools were not altered significantly in the presence of 2 mM HU, whereas dCTP and dGTP pools were 50 and 70% of that measured in virus infected cells unexposed to the drug.

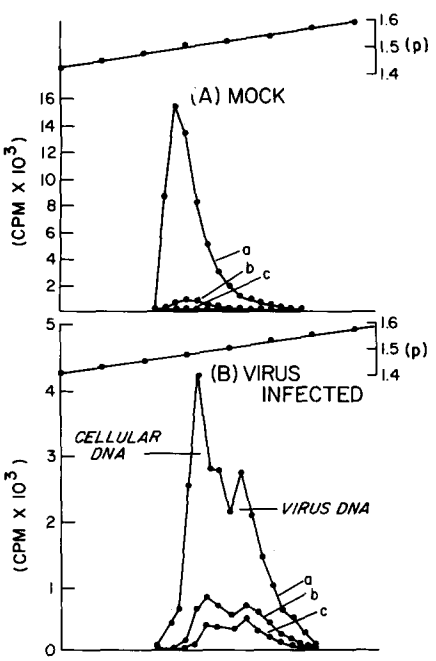

Fig. 3. [^{32}P]incorporation into DNA. Isopycnic centrifugation of DNA from mock (A) and HSV-2 infected (B) HeLa S3 cells in the absence (a) or the presence of 1 mM HU (b) and 2 mM HU (c). (p) denotes gradient density in g/cm^3. For details, see Experimental Procedures.

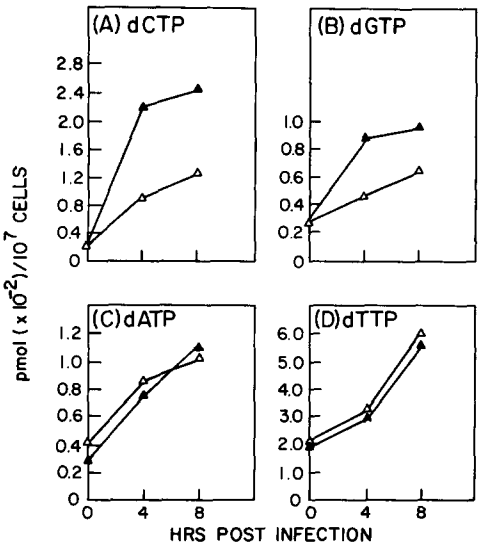

Fig. 4. dNTP pools in HSV-2 infected cells. dNTPs were determined in neutralized perchloric extracts prepared at 0, 4 and 8 hr post infection in the absence (▲) or the presence of 2 mM HU (△). For details, see Experimental Procedures.

Effect of hydroxyurea on virus yield. The antiviral activity on HU was examined in three independent yield reduction assays using culture medium supplemented with dialyzed serum as described earlier. As can be seen in Table 1, 16% of the infectious virus yield was observed in the presence of 2 mM HU as compared to the control which received no drug.

DISCUSSION

Ribonucleotide reductase is a critical enzyme in providing deoxynucleotides for cellular DNA synthesis. It is unclear how critical the role of virus induced ribonucleotide reductase is in providing deoxynucleotides for HSV DNA synthesis. Studies by others [13], using temperature-sensitive virus mutants, suggested that virus ribonucleotide

Table 1. Effect of HU on HSV-2 yield*

HU (mM)	% of Control
0	100
1.0	30 ± 7.6
2.0	16.4 ± 7.3

* Values are expressed as percent yield (mean ± S.D.) of a control receiving no drug. At least three determinations were made for each value.

reductase may be essential for viral replication since virus yield was low and the activity of ribonucleotide reductase was undetectable at nonpermissive temperatures. However, in the same study, 20% of the virus DNA synthesis at the permissive temperature was observed at the nonpermissive temperature. This raises the question of whether the inability of the HSV mutant (tsG) to grow well at nonpermissive temperatures resulted from other lesions of the mutant, related or unrelated to the ribonucleotide reductase lesion. Furthermore, even if ribonucleotide reductase is the major lesion of the mutant responsible for its inability to grow well at nonpermissive temperatures, virus ribonucleotide reductase protein may have other roles than providing deoxyribonucleotides for virus DNA synthesis. The demonstration of undetectable ribonucleotide reductase activity in tsG infected cells at nonpermissive temperatures does not provide evidence that the activity is critical.

The approach this laboratory took in examining the role of ribonucleotide reductase activity in virus replication is to take advantage of hydroxyurea as an equally potent inhibitor of host and virus ribonucleotide reductase, as shown in Fig. 1. These results are consistent with observations made by others [14, 15]. When 2 mM hydroxyurea was employed in cell culture studies, more than 95% of the incorporation of [^{14}C]Cyd into DNA in virus infected cells was inhibited without inhibition of the total uptake of [^{14}C]Cyd into RNA, suggesting that the intracellular ribonucleotide reductase activity, whether host or virus specified, was inhibited. However, the deoxynucleotide pools in hydroxyurea-treated infected cells were found not to be affected by more than 2-fold when compared with their controls. The phenomenon of deoxynucleotide pool expansion in virus infected cells as reported previously [16] was observed in spite of the presence of hydroxyurea (Fig. 4). These results suggested that ribonucleotide reductase may not be solely responsible for providing deoxynucleotides for DNA synthesis, unless less than 5% of the ribonucleotide reductase activity is sufficient to maintain the intracellular pools of deoxyribonucleotides as observed. If this is the case, then to have an effective inhibitor of HSV targeted at viral RR, 95% inhibition of intracellular RR activity must be achieved, whether this activity is of viral or host origin. Since these studies were performed in culture medium containing dialyzed serum, the deoxynucleotide pools have to come from sources *de novo*. It was suggested that host DNA breakdown could be a reasonable source [1]. We are currently investigating this possibility.

When DNA synthesis was examined using [^{32}P], HU at 2 mM could inhibit more than 98% of the [^{32}P]incorporation into DNA of mock infected cells, whereas only 75% was inhibited in virus infected cells. The inhibition of virus DNA synthesis by 2 mM HU is consistent with the inhibition of virus yield (Table 1). There were other reports which indicated the IC_{50} of HU against HSV-1 growth to be around 0.25 mM [17]. Since the concentration range of HU reported in that study was 1–4 mM, it is unsure how linear the dose–response curve between 0 and 1 mM was, which is required for the establishment of an IC_{50}. Our unpublished results indicate a nonlinear dose–response curve in that range.

The inhibition of 75% of the virus DNA synthesis in the present study could be due to the decreased dCTP (50%) and dGTP (30%) intracellular pools, which could be the consequence of inhibition of intracellular ribonucleotide reductase activity. It is also a possible result of inhibition of some other DNA synthetic steps by HU. Indeed, the suggestion has been made that HU at high concentrations may affect other functions such as DNA processing, or ribonucleotide reductase could have another function in cells unrelated to dNTP provision [18].

In summary, routes other than the conventional *de novo* or salvage pathways of dNTP formation are operating in virus infected cells. At least 95% of the available ribonucleotide reductase activity is not essential for virus replication. Thus, using ribonucleotide reductase as a target for developing antiherpes agents may not be a fruitful approach. The use of agents targeted at ribonucleotide reductase in conjunction with compounds which inhibit other dNTP forming pathways may, however, be beneficial.

Note added in proof: A detailed characterization of the antibodies used in this study and provided by Dr. Bacchetti are described in: Huszar *et al.*, *J. gen. Virol.* **64**, 1327 (1983), and Bacchetti *et al.*, *J. Virol.* **49**, 591 (1984).

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