Deoxyadenosine Reverses Hydroxyurea Inhibition of Vaccinia Virus Growth

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Hydroxyurea, an inhibitor of ribonucleotide reductase, blocks replication of vaccinia virus. However, when medium containing hydroxyurea and dialyzed serum was supplemented with deoxyadenosine, the block to viral reproduction was circumvented, provided that an inhibitor of adenosine deaminase was also present. Deoxyguanosine, deoxycytidine, and deoxythymidine were ineffective alone and did not augment the deoxyadenosine effect. In fact, increasing concentrations of deoxyguanosine and deoxythymidine, but not deoxycytidine, eliminated the deoxyadenosine rescue, an effect that was reversed by the addition of low concentrations of deoxycytidine. These results suggested that the inhibition of viral replication by hydroxyurea was primarily due to a deficiency of dATP. Deoxyribonucleoside triphosphate pools in vaccinia virus-infected cells were measured at the height of viral DNA synthesis after a synchronous infection. With 0.5 mM hydroxyurea, the dATP pool was >90% depleted, the dCTP and dGTP pools were 40 to 50% reduced, and the dTTP pool was increased. Assay of ribonucleotide reductase activity in intact virus-infected cells suggested that hydroxyurea may differentially affect reduction of the various substrates of the enzyme.

The ability of hydroxyurea (HU) and related hydroxamic acids to preferentially inhibit DNA metabolism was initially described more than 25 years ago (54). HU-treated cells are reversibly arrested in the early S phase, but prolonged exposure results in cytotoxicity, leading to cell death (5, 14, 50). Although neither the acute nor long-term effects are fully understood, HU continues to be used clinically as an antitumor agent (4).

The rate-limiting enzyme in deoxynucleoside triphosphate (dNTP) biosynthesis, ribonucleotide reductase (EC 1.17.4.1), is the immediate target of HU (37), and the site of HU action has been localized to the smaller of the two subunits of the enzyme (20). Inhibition is thought to result from the ability of the drug to destroy a stable organic free radical carried on a tyrosine residue that is invariant in small reductase subunits from sources as diverse as *Escherichia coli*, yeast species, mice, and vaccinia virus (35, 40). Although mechanistic details of the reaction catalyzed by ribonucleotide reductase remain obscure, the tyrosyl radical has been proposed to initiate reduction of the ribonucleotide substrates (36, 46).

If HU acts by starving the replication apparatus for precursors, then the block should be reversed if dNTPs are supplied as exogenous deoxyribonucleosides, just as thymidine can relieve inhibition of DNA synthesis by fluorodeoxyuridine, an inhibitor of thymidylate synthase. Nevertheless, numerous attempts to reverse HU inhibition have yielded preponderantly negative results (2, 8, 11, 16, 21, 30, 49, 53, 55), and several of the reported restorations of DNA synthesis can be ascribed to changes in [³H]dTTP specific activity (34). These and other observations (5, 52) have led to the suggestion that HU may have additional sites of action (1, 37, 53) or that certain precursor pools derived by salvage pathways are excluded from replication forks (23, 34, 42).

Although no additional primary targets for HU have been identified, and HU-resistant cell and viral mutants all contain either an altered or amplified ribonucleotide reductase small subunit (for a review, see reference 25), the cytotoxic effects of the drug or its metabolites (e.g., chromosome breakage [19] and membrane damage [22]) may preclude effective rescue of cell growth from HU. To circumvent possible complications due to secondary effects, we have reexamined the reversal of HU-inhibition by using vaccinia virus, which encodes ribonucleotide reductase subunits that are highly homologous to their mammalian counterparts (32, 40, 47). Vaccinia virus reproduction is inhibited by HU with a 50% effective concentration of 0.5 to 0.7 mM, and the virus-encoded small subunit is presumed to be a target for the drug, since resistant mutants with amplified genes for this protein have been isolated (41).

We found that HU-mediated inhibition of vaccinia virus reproduction was reversible by exogenous deoxyribonucleosides, but only if an inhibitor of adenosine deaminase was present. Surprisingly, deoxyadenosine (dAdo) alone was as effective as a mixture of all four deoxyribonucleosides. The implications of these results for understanding the interaction of HU with ribonucleotide reductase are discussed.

MATERIALS AND METHODS

Materials. [2,8-³H]adenosine ([³H]Ado; 39 Ci/mmol) and [5-³H]cytidine ([³H]Cyd; 25 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, Calif.). [8-³H]guanosine ([³H]Guo; 11 Ci/mmol) was a gift from Buddy Ullman, Oregon Health Sciences University. [2,8-³H]ADP (25 Ci/mmol), [5-³H]CDP (25.3 Ci/mmol), and [8-³H]GDP (5 Ci/mmol) were obtained from DuPont (NEN Research Products, Boston, Mass.). Erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA) was donated by Burroughs Wellcome Co. (Research Triangle Park, N.C.). HU and calf intestinal phosphatase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). dNTPs, poly(dA-dT), and poly(dI-dC) used in the dNTP assays were purchased from

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Pharmacia (Piscataway, N.J.). PolI (Klenow fragment) was obtained from United States Biochemical (Cleveland, Ohio). All other biochemicals were purchased from Sigma (St. Louis, Mo.).

Cells and virus. BSC₄₀ monkey kidney cells and the WR strain of vaccinia virus were maintained as described previously (38), except that virus stocks were subjected to two freeze-thaw cycles rather than trypsinization before infections were initiated. Periodic screening for mycoplasma with Hoechst 33258 dye revealed no evidence of infection. Confluent cell monolayers received fresh medium containing 5% dialyzed fetal calf serum (GIBCO) 16 h before the beginning of an experiment and again at the time of infection. For plaque reduction assays, cells were infected with vaccinia virus at a dilution that yielded 100 to 300 plaques per dish in the absence of drugs or nucleosides. Plaques were visualized by staining monolayers with 0.5% (wt/vol) methylene blue in 50% methanol. In most experiments, triplicate dishes were used for each data point. Except where plaque numbers were very low, the standard deviation was <15% of the mean value.

Analysis of dNTP pools. Confluent monolayers of BSC₄₀ cells in 100-mm dishes were infected with vaccinia virus (multiplicity of infection, 10). Triplicate dishes were prepared for each data point. Treatments (HU and/or deoxyribonucleosides) were initiated at 3.5 h postinfection (hpi) and terminated at 4.5 hpi by aspirating medium and rapidly washing the monolayers twice with 2 ml of cold Tris-buffered saline (10 mM Tris-HCl [pH 8], 150 mM NaCl). Nucleotides were extracted into 2 ml of 60% methanol at -20°C for 1 h. After insoluble material was pelleted, extracts were taken to dryness and suspended in 5% trichloroacetic acid (100 µl per 100-mm dish). After 30 min on ice, extracts were centrifuged to remove insoluble material, and supernatants were extracted with an equal volume of 0.5 M tri-*n*-octylamine-78% Freon. dNTPs were quantitated by the enzymatic method, essentially as described previously (29).

Analysis of ribonucleotide pools. Portions of the extracts prepared for dNTP analysis were analyzed on a Partisil-10 SAX high-pressure liquid chromatography ion-exchange column (Whatman, Clifton, N.J.) exactly as described previously (24). Peaks were detected by A_{254} and identified by their retention times as compared with those of standards.

Assay of ribonucleotide reductase activity. For *in vitro* assays, vaccinia virus ribonucleotide reductase was partially purified from virus-infected cells, and CDP reduction was measured as described previously (38, 39). For measurement of ADP and GDP reduction, reaction mixtures (20 µl) contained 100 mM 4-(2-hydroxyethyl)-1-piperazine-*N'*-2-ethanesulfonic acid (pH 8), 10 mM dithiothreitol, 4 mM adenylylimido diphosphate, 2 mM magnesium acetate, 20 µM FeCl₃, 50 µM tritiated substrate (specific activity, 100 cpm/pmol), and effector nucleotides at various concentrations. Reactions were stopped by heating at 100°C. Nucleotides were dephosphorylated by calf intestinal phosphatase (1 U per reaction, 37°C, 2 h), and reaction products were chromatographed as described previously (33) and quantitated by scintillation counting.

To measure *in situ* ribonucleotide reductase activity, we adapted a method previously used in mammalian cells to estimate CDP reduction after HU treatment (6). Infected cell monolayers (35-mm dishes; multiplicity of infection, 10 to 15) were treated with 0 to 5 mM HU at 3.5 hpi and then exposed from 4 to 5 hpi to a trace amount (0.3 µM) of radioactive ribonucleoside precursor ([³H]Ado, [³H]Guo, or [³H]Cyd). Methanol-soluble molecules were extracted from

the cells, and radioactivity in deoxyribonucleosides and deoxyribonucleotides was quantitated by using the same chromatography systems utilized for *in vitro* assays. The methanol-insoluble fraction was digested with 0.3 N NaOH to hydrolyze RNA, and radioactivity incorporated into DNA (base-resistant, acid-precipitable material) was collected on GF/C filters. Radioactivity incorporated into DNA in the presence of 0.1 M cytosine β-D-arabinoside was taken as background. This cytosine β-D-arabinoside concentration is 50-fold greater than the level required to eliminate vaccinia virus plaque formation. To quantitate extracellular radioactivity in deoxyribonucleosides, a portion of the medium was concentrated and analyzed.

In preliminary experiments to characterize this method in the vaccinia virus-infected cell system, cultures were harvested 0, 30, 60, and 90 min after the addition of labeled compounds. The rate of uptake of [³H]Ado and [³H]Cyd into the methanol-soluble fraction was not affected by vaccinia virus infection. [³H]Cyd reached isotope equilibrium after 30 to 60 min, whereas [³H]Ado did not achieve equilibrium even after 90 min. This difference can be attributed to the ATP pool being much larger than the CTP pool. The acid-precipitable radioactivity present after base digestion was 3 to 5% of the total radioactivity present in the methanol-insoluble fraction.

RESULTS

Inhibition of adenosine deaminase potentiates dAdo toxicity. Before attempting to circumvent HU effects on vaccinia virus growth, we addressed the problem of dAdo diversion resulting from the action of the salvage enzyme adenosine deaminase (30). Using dAdo toxicity to virus growth as an assay (a phosphorylated metabolite of dAdo, dATP, inhibits the activity of ribonucleotide reductase toward all four of its substrates, leading to a depression of DNA synthesis as dATP accumulates), we asked whether the adenosine deaminase inhibitor EHNA was effective in counteracting diversion of dAdo (17) in the vaccinia virus-infected cell. EHNA decreased the 50% effective concentration of dAdo from 360 µM (0 µM EHNA) to 60 µM (5 to 10 µM EHNA). In subsequent experiments we included EHNA at 5 µM whenever dAdo was added to the medium, unless otherwise noted.

HU-mediated inhibition of plaque formation can be reversed by deoxyribonucleosides and EHNA. To see whether exogenous deoxyribonucleosides could reverse HU inhibition of vaccinia virus growth, plaque assays were conducted under the various conditions indicated in Fig. 1. Only one combination of medium supplements tested—10 µM each dAdo, deoxyguanosine (dGuo), deoxycytidine (dCyd), and deoxythymidine (dThd) and 5 µM EHNA—supported plaque formation in the presence of 2 mM HU. Under these conditions, the number of plaques per dish was 75% of the control value (no HU or deoxynucleoside additions). Without EHNA, neither 10 nor 50 µM deoxyribonucleoside was effective as a supplement. The higher concentration of precursors appeared to potentiate the inhibition by HU, whether EHNA was present or not. It was also apparent that, in the absence of HU, the combination of EHNA plus 50 µM deoxyribonucleosides was inhibitory.

Reversal of HU inhibition is a function of dAdo concentration. To determine the concentration of each of the four deoxyribonucleosides that maximized reversal of HU inhibition, cells were infected with virus in the presence of HU (2 mM), EHNA (5 µM), and three of the four deoxyribonu-

FIG. 1. Effect of deoxyribonucleosides and EHNA on HU-mediated inhibition of plaque formation. Symbols: ○, control; ●, 5 μ M EHNA; △, 10 μ M dAdo, dGuo, dCyd, and dThd; ▲, 50 μ M dAdo, dGuo, dCyd, and dThd; □, 5 μ M EHNA, 10 μ M dAdo, dGuo, dCyd, and dThd; ■, 5 μ M EHNA, 50 μ M dAdo, dGuo, dCyd, and dThd. Results from three dishes were averaged for each data point.

FIG. 3. Effect of HU on vaccinia virus growth in the absence (○) or presence (●) of dAdo (10 μ M) plus EHNA (5 μ M) or in the presence all four deoxyribonucleosides (10 μ M each) plus EHNA (△). Monolayers of BSC₄₀ cells were infected at a multiplicity of infection of 0.1 and harvested after 24 h; titers were determined by plaque assay.

cleosides (10 μ M). The fourth precursor was added at concentrations ranging from 0 to 50 μ M. The unexpected result was that restoration of plaque formation was solely a function of the dAdo concentration (Fig. 2A). Furthermore, dAdo was effective only at concentrations below 30 μ M. The finding that the efficiency of plaque restoration did not change as dCyd, dThd, and dGuo were varied suggested that their presence was not essential. This was confirmed by the experiment shown in Fig. 2B. Conditions were identical to those in Fig. 2A, except that only one deoxyribonucleoside precursor was added to each set of cultures. The results clearly demonstrated the potency of dAdo at 3 to 10 μ M, but only in combination with EHNA. The maximum rescue efficiency achieved by dAdo plus EHNA was 73%.

A more sensitive measure of HU inhibition and its reversal is provided by a two-step growth assay—a protocol in which the agents are present during several cycles of viral reproduction in culture, followed by titration of total infectious

virus by plating in the absence of the inhibitor. The ability of dAdo alone to support virus growth in the presence of HU was compared with the effect of all four precursors. HU alone depressed virus reproduction by 3 orders of magnitude at 5 mM (Fig. 3). Both treatments supported a 13-fold increase in viral reproduction in the presence of 2 mM HU and a 60- to 90-fold increase in the presence of 5 mM HU.

dAdo-mediated reversal of HU inhibition can be eliminated by dThd and dGuo. One explanation for the effectiveness of dAdo in counteracting HU is that the drug affects vaccinia virus replication primarily by inhibiting ADP reduction, leading to a critical depletion of the dATP pool. To investigate the activity of ribonucleotide reductase toward its pyrimidine-containing substrates under dAdo rescue conditions, we took advantage of the allosteric regulatory properties of the enzyme. As isolated from both *E. coli* and mammalian cells, the enzyme is inhibited in the reduction of CDP or UDP by either dTTP or dGTP, presumably because

FIG. 2. (A) Effect of varying the concentration of each deoxyribonucleoside on HU-mediated inhibition of plaque formation. HU was present in all dishes at 2 mM, EHNA was present at 5 μ M, and three of the four deoxyribonucleosides were present at 10 μ M. The concentration of the fourth deoxyribonucleoside is indicated on the x axis. Symbols: ○, dAdo varied; ●, dCyd varied; △, dGuo varied; ▲, dThd varied. The control value (no HU) was 63 plaques per dish. Results from three dishes were averaged for each data point. (B) Effect of single deoxyribonucleosides on HU-mediated inhibition of plaque formation. HU was present in all dishes at 2 mM. Symbols: ○, dAdo plus EHNA (5 μ M); ●, dAdo; △, dCyd; ▲, dGuo; □, dThd. Control value (no HU) was 141 plaques per dish. Each data point is the average of three determinations.

FIG. 4. (A) Effects of dGuo (○), dThd (●), and dCyd (△) on reversal of HU inhibition by dAdo plus EHNA. HU (2 mM), dAdo (10 μM), and EHNA (5 μM) were present in all dishes. The control value (no HU) was 54 plaques per dish. Each data point is the average of three determinations. (B) Reinstatement by dCyd of reversal of HU inhibition. HU, dAdo, and EHNA at the concentrations indicated for panel A were present in all dishes. The effects of increasing concentrations of dGuo with (●) or without (○) dCyd (10 μM) are plotted.

these effectors play positive allosteric roles in GDP or ADP reduction, respectively (7, 12). We first determined that vaccinia virus-encoded ribonucleotide reductase is regulated in a manner similar to that of the well-characterized *E. coli* and mammalian enzymes in that dTTP and dGTP inhibited CDP reduction but stimulated GDP and ADP reduction, respectively (data not shown). Although these experiments were done with crude extracts, the activities measured almost certainly represent the virus-encoded enzyme, since the level of CDP reductase activity exceeded that in extracts from uninfected cells by 10-fold. Additionally, it has recently been shown that the increase in ribonucleotide reductase activity seen after vaccinia virus infection is dependent on an intact viral large subunit gene (9). If the dAdo-mediated reversal of HU inhibition were dependent on continued reduction of pyrimidine substrates, therefore, the reversal should be sensitive to dThd or dGuo added to the culture medium. This was tested in the experiment shown in Fig. 4A, in which the addition of either dThd or dGuo to the medium resulted in a dose-dependent elimination of the reversal. The dAdo-mediated reversal, however, was insensitive to exogenous dCyd; dCTP has no known role in regulating ribonucleotide reductase (12). To obtain evidence that the negative effects depicted in Fig. 4A were, in fact, due to depletion of the dCTP and/or dTTP pools, we added a low concentration of dCyd to cultures containing HU, dAdo, EHNA, and various concentrations of dGuo. Indeed, 10 μM dCyd restored circumvention of the HU block (Fig. 4B). The difference in mean plaque number in cultures with and without dCyd in the absence of any dGuo was not statistically significant (means $\pm$ standard deviations, 298 ± 39 and 250 ± 34 , respectively).

Effects of HU on dNTP pools in virus-infected cells. To determine the effects of HU on DNA precursor pools during the period of active viral replication, we measured dNTP levels 1 h after the addition of various concentrations of HU. The dATP pool was most dramatically affected by HU; it was depleted to 10% of the untreated level by 0.5 mM HU (Fig. 5). Changes in the dGTP, dCTP, and dTTP pools were more moderate and were continuous over the range of HU concentrations tested. The dGTP pool (the smallest in the absence of HU) and the dCTP pool were both reduced to about half of control values by 0.5 mM HU and to about one-third of control values by 2 mM HU. The dTTP pool nearly doubled in the HU range from 0 to 5 mM.

Replenishment of the dATP pool by the addition of dAdo and EHNA. To further explore the mechanism by which dAdo and EHNA rescued vaccinia virus from HU inhibition, we measured the effects of these compounds on dNTP pool sizes in the presence of various concentrations of HU. As anticipated, dAdo elevated the dATP pool, and this pool remained elevated at all HU concentrations tested (Fig. 6). Additionally, dAdo appeared to moderate the HU-induced increase in the dTTP pool and augment the drug-mediated decrease in the dGTP pool. However, since the conditions imposed in the Fig. 6 experiment are those that most effectively rescue virus reproduction, the observed changes in the dTTP and dGTP pools may not be a direct result of the dAdo supplementation but secondary to the demands of ongoing DNA synthesis.

HU-mediated inhibition of the flow of tritiated ribonucleo-

FIG. 5. Effect of HU on dNTP pools in vaccinia virus-infected cells. Pool sizes were measured 1 h after the addition of HU to the concentrations indicated. HU was added 3.5 hpi; the multiplicity of infection was 10. Error bars indicate standard deviations for triplicate dishes.

FIG. 6. Effect of HU on dNTP pools in the presence of dAdo (10 μ M) and EHNA (5 μ M). Experimental details were as described in the legend to Fig. 5.

sides into DNA and deoxyribonucleoside and deoxyribonucleotide pools. Since all four substrates of ribonucleotide reductase (ADP, GDP, CDP, and UDP) are thought to be reduced in the same catalytic site by a mechanism involving the same HU-sensitive radical, differential inhibition of the four reactions catalyzed by this enzyme was unexpected. To more directly examine the effects of HU on ribonucleotide reduction in the vaccinia virus-infected cell, we measured the relative activity of the enzyme with respect to three of its four substrates by using a protocol based on measurement of the enzyme activity in uninfected cells (6). In this method, cells are exposed to a tracer amount of tritiated ribonucleo-

side ($[^3\text{H}]\text{Ado}$, $[^3\text{H}]\text{Guo}$, or $[^3\text{H}]\text{Cyd}$); after a period of time, intra- and extracellular fractions containing deoxyribonucleoside and deoxyribonucleotide derivatives of the precursors are analyzed. Summation of the radioactivity that has passed through ribonucleotide reductase during the labeling period yields an estimate of the relative activity of the enzyme under various conditions.

Incorporation of all three precursors into DNA was profoundly inhibited by 0.5 mM HU and was almost undetectable after treatment with 2 or 5 mM HU (Table 1). In contrast to the effects of HU on the incorporation of ribonucleoside precursors into DNA, however, the drug exhibited an apparent differential effect on precursor incorporation into deoxyribonucleoside and deoxyribonucleotide pools. HU at 0.5 mM decreased radioactivity flowing into $[^3\text{H}]\text{Ado}$ -, $[^3\text{H}]\text{Guo}$ -, and $[^3\text{H}]\text{Cyd}$ -derived deoxy pools by 96, 63, and 49%, respectively. Label derived from radioactive Cyd was present in both dCyd- and dUrd-containing nucleotides at about a 1:1 ratio whether HU was present or not (data not shown), suggesting that deamination of dCyd-containing compounds was not affected by HU. Cytosine β -D-arabinoside (0.1 mM) had no effect on accumulation of radioactivity into any deoxyribonucleoside or deoxyribonucleotide pool (data not shown), indicating that the observed effects were not simply secondary to cessation of DNA synthesis. Although excretion of purine deoxyribonucleosides was not detected, more than half of the total radioactivity in the deoxy fractions derived from $[^3\text{H}]\text{Cyd}$ was present as extracellular $[^3\text{H}]\text{dCyd}$ - $[^3\text{H}]\text{dUrd}$. The excretion and reutilization of pyrimidine deoxyribonucleosides in mammalian cells have been well characterized (27). Summing the radioactivity in the various fractions yielded the data shown in the right-hand column of Table 1. The apparent relative sensitivity to HU at all concentrations of the drug tested was ADP reduction > GDP reduction >> CDP reduction.

Although HU is reported to have little or no effect on RNA metabolism (53), and our preliminary labeling studies indi-

TABLE 1. Relative effects of HU on in situ ribonucleotide reductase activity^a

Precursor	HU (mM)	cpm per 35-mm dish $\pm$ SD			
		DNA	Intracellular deoxy pools	Extracellular deoxy pools	Total (%)
$[^3\text{H}]\text{Cyd}$	0	2,418 $\pm$ 178	3,147 $\pm$ 303	7,897 $\pm$ 1,389	13,462 (100)
	0.5	174 $\pm$ 45	1,997 $\pm$ 133	3,587 $\pm$ 756	5,758 (43)
	1.0	129 $\pm$ 51	1,570 $\pm$ 153	2,743 $\pm$ 309	4,442 (33)
	2.0	3 $\pm$ 24	1,547 $\pm$ 253	2,583 $\pm$ 476	4,133 (31)
	5.0	0	1,850 $\pm$ 273	2,243 $\pm$ 335	4,093 (30)
$[^3\text{H}]\text{Ado}$	0	5,261 $\pm$ 442	1,033 $\pm$ 180	ND	6,294 (100)
	0.5	204 $\pm$ 41	46 $\pm$ 29		250 (4)
	1.0	10 $\pm$ 36	51 $\pm$ 39		61 (1)
	2.0	0	29 $\pm$ 108		29 (<1)
	5.0	0	27 $\pm$ 68		27 (<1)
$[^3\text{H}]\text{Guo}$	0	546 $\pm$ 82	112 $\pm$ 26	ND	658 (100)
	0.5	76 $\pm$ 38	41 $\pm$ 12		117 (18)
	1.0	53 $\pm$ 21	20 $\pm$ 28		73 (11)
	2.0	17 $\pm$ 5	23 $\pm$ 24		40 (6)
	5.0	56 $\pm$ 34	24 $\pm$ 23		80 (12)

^a Cultures infected with vaccinia virus (multiplicity of infection, 15) were labeled from 3.5 to 4.5 hpi with the indicated compounds (0.3 μ M). The radioactivity in DNA is methanol-insoluble, base-resistant, acid-precipitable radioactivity. Background values (subtracted) were determined in the presence of 0.1 mM cytosine β -D-arabinoside (113 cpm for $[^3\text{H}]\text{Cyd}$, 260 cpm for $[^3\text{H}]\text{Ado}$, 13 cpm for $[^3\text{H}]\text{Guo}$). Values tabulated are the mean of determinations made on three independent 35-mm dishes of infected cells. ND, Not detected.

FIG. 7. Nucleotide pools in vaccinia virus-infected cells at 4.5 hpi determined from high-pressure liquid chromatography. Upper tracing, control (90,148 total arbitrary absorbance units); lower tracing, 1 h of treatment with 5 mM HU (96,330 total arbitrary absorbance units).

cated no significant effect of the drug on incorporation of precursors into RNA (data not shown), we wished to determine directly whether HU altered ribonucleotide pool sizes, since such effects could cause differential changes in the specific activities of ribonucleotide reductase substrates. Methanol-soluble compounds extracted from vaccinia virus-infected cells were resolved by chromatography on high-pressure liquid chromatography anion-exchange column (24), and the percentage of the total absorbance present in each peak was calculated. Comparison of the values obtained after a 1-h treatment with 0, 2, or 5 mM HU revealed that there was no significant effect of the drug on any NMP or NTP pool, as shown in the representative tracings from 0 and 5 mM HU treatments shown in Fig. 7. Additionally, the

pyrimidine nucleoside diphosphate pools were not detectably altered. However, treatment with either 2 or 5 mM HU resulted in a twofold increase in the size of the peaks migrating at the positions of GDP and ADP. From this analysis, we concluded that the specific activity of [3 H]CDP was unaltered but that the specific activities of [3 H]ADP and [3 H]GDP may have decreased approximately twofold after treatment with 2 to 5 mM HU. Changes of this magnitude, however, would not alter the conclusions we have drawn from the data in Table 1. The apparent increase in ADP and GDP pools observed after 1 h of HU treatment suggests that the drug block results in accumulation of ribonucleotide reductase substrates. Additional characterization of these peaks will be required before definitive conclusions can be drawn.

DISCUSSION

In the present study we report the novel and unexpected finding that HU inhibition of vaccinia virus growth was circumvented by the addition of dAdo to the growth medium. Although partial reversal of HU-inhibited *Tetrahymena pyriformis* growth was achieved by adding the four deoxyribonucleoside precursors to the medium (8), reversal of growth inhibition in mammalian cells has not been reported. The conditional nature of the effects of the drug in the vaccinia virus system allowed us to explore several parameters of HU action, and the results lead us to suggest that HU inhibition of ribonucleotide reductase *in vivo* may be more complex than previously assumed.

We considered various hypotheses that might account for our finding that dAdo reversed HU inhibition of virus growth. Since resistance to HU can be mediated by elevated levels of the target enzyme (resulting most commonly from gene amplification), we asked whether dAdo might act by increasing the amount of either subunit of ribonucleotide reductase produced during a vaccinia virus infection. As measured by immunoblotting and enzyme assays, there was no such effect (data not shown). We next considered the possibility that exogenously added dAdo provided a source of deoxyribose that was then recycled by purine nucleoside phosphorylase to supplement all four DNA precursor pools. Several results of our study, however, conflict with this hypothesis. EHNA, a potent inhibitor of adenosine deaminase, counteracts the conversion of dAdo to deoxyinosine. Since deoxyinosine is a much better substrate than dAdo for purine nucleoside phosphorylase (45), the recycling hypothesis predicts that EHNA would inhibit the dAdo rescue effect. Instead, we found that EHNA was required to achieve the rescue effect, suggesting a specificity for dAdo-containing compounds. Additionally, dGuo, which is efficiently degraded and recycled by purine nucleoside phosphorylase, was ineffective as a rescue agent. The third hypothesis we considered is that HU inhibits preferentially the reduction of purine substrates, especially the reduction of ADP, and that dAdo relieves inhibition by replenishing directly the dATP pool and, perhaps indirectly, the dGTP pool. Three lines of evidence are consistent with this hypothesis.

(i) Elimination of the dAdo rescue effect by dThd and dGuo. These latter two deoxyribonucleosides are precursors of compounds that serve as allosteric regulators of ribonucleotide reductases extracted from *E. coli* and mammalian cells (7, 12) and from vaccinia virus-infected cells (this study). The simplest explanation of the results shown in Fig. 4 is that the reduction of pyrimidine substrates is occurring at a rate

sufficient to support viral replication, albeit at a reduced level, in the presence of HU and dAdo, and that this residual reduction can be eliminated by the allosteric effectors dTTP and dGTP. Of course, these results do not eliminate the possibility that dThd and dGuo, or their metabolites, deplete the dCTP pool by some as-yet-undescribed mechanism.

(ii) **dNTP pool studies.** The differential pattern of dNTP pool changes we observed in HU-treated, vaccinia virus-infected cells was strikingly similar to that reported by a number of other investigators using a variety of cell types (3, 6, 10, 11, 21, 26, 37, 42, 51). We found the dATP pool to be depressed even more severely than what is usually reported, but this may be due to the fact that in our experiments essentially all of the cells were engaged in (viral) DNA synthesis, whereas most studies have been conducted on unsynchronized cell populations. However, the concentration of HU that caused a 90% depletion of the dATP pool (0.5 mM) effected only a 50% inhibition of virus growth. This anomaly is likely due to the different time scales of the two types of experiment. The biological response of vaccinia virus to agents that inhibit DNA synthesis is abnormally prolonged transcription and translation of early genes, a class that includes both ribonucleotide reductase genes. The presence of HU during 24 to 48 h of virus growth thus leads to overproduction of ribonucleotide reductase, which eventually confers partial drug resistance on viral replication (39).

(iii) **Measurement of in situ ribonucleotide reductase activity.** To determine more directly whether HU differentially affects reduction of purine versus pyrimidine substrates, we estimated relative ribonucleotide reductase activity in the presence of increasing concentrations of HU by summing radioactivity present in deoxyribose-containing molecules (DNA plus intra- and extracellular pools) labeled from ribonucleoside precursors added to the cell growth medium. The accumulation of radioactivity into total intracellular methanol-soluble and -insoluble pools was not significantly affected by HU; however, inhibition of the flow of label into dAdo-containing acid-soluble pools paralleled the decrease in DNA labeling we observed with each of the three precursors; the radioactivity flowing into labeled dGuo- and dCyd-containing acid-soluble pools was less markedly affected. In these experiments, we attempted to account for all significant metabolic fractions into which products of ribonucleotide reductase might flow; however, we were unable to measure conversion of dUMP to dTMP by thymidylate synthase by measuring tritiated water in the cell culture medium (31) because of a high background contributed by the [^3H]Cyd label. Without knowing what fraction of the total dTTP pool is derived from dCTP in vaccinia virus-infected cells, we cannot estimate how much of the total CDP reduction output was lost from our analysis by this omission. Nevertheless, since thymidylate synthase is insensitive to feedback inhibition by elevated dTTP levels (18), this likely resulted in an underestimate of CDP reduction in the presence of HU. A metabolic fate of the dCTP pool unrelated to DNA synthesis, incorporation into liponucleotides, utilizes 10 to 20% of this pool in some cell types (13, 43, 44). Any [^3H]dCTP so incorporated in the present study would have been included in the thin-layer chromatographic quantitation of intracellular [^3H]dCMP after sample preparation by acid hydrolysis (see Materials and Methods).

A differential effect of HU on purine versus pyrimidine substrate reduction has not been demonstrated in vitro. Nevertheless, several authors have presented indirect evidence that reduction of purines, especially ADP, is more sensitive to HU than reduction of pyrimidines (reviewed in

reference 25). A partial reversal of HU-induced pool changes and DNA synthesis inhibition was recently reported in hamster lung fibroblasts after combined treatment with dAdo (plus EHNA) and dGuo (21). Data obtained from isotope dilution analysis of rat thymus cells led to the suggestion that the increased dTTP pool observed after HU treatment resulted from residual function of ribonucleotide reductase (34). Additionally, evidence of a differential effect of HU on ribonucleotide reduction was presented in a 1973 study of Chinese hamster cells (51). Synchronized by mitotic shake-off and then exposed to HU during the G1 phase of the cell cycle, these cells accumulated dTTP, dCTP, and dGTP but not dATP as they approached the time they would normally have initiated DNA synthesis. Therefore, although we have studied a virus-infected cell system, it seems likely that our results are relevant to an understanding of the effects of HU on cellular replication.

That HU destroys the proteinaceous free radical of all known iron-containing ribonucleotide reductases has been widely documented. However, the concentration of HU required in vitro to yield a certain inhibition and the conditions required for regeneration of enzyme activity vary widely. Analyses that depend on assay of enzyme activity may be complicated by spontaneous regeneration of the radical under the usual reaction conditions employed (e.g., air, iron, dithiothreitol) (48), and the recent demonstration of a strong pH dependence of the regeneration reaction in vitro may provide a partial explanation for the differing results reported previously (15).

Although we have interpreted the results of the present study as most consistent with a differential susceptibility of ribonucleotide reductase to HU, a mechanistic explanation in light of the accepted model of HU action is not readily apparent. Substrate specificity is governed by allosteric effector sites on the large subunit of the enzyme, whereas HU sensitivity is a property of the small subunit. The structure of the *E. coli* small subunit was recently determined and revealed that the tyrosine residue on which the HU-sensitive radical resides is buried within the protein some 1.0 nm from the nearest surface. The possibility of electron transfer between the iron-tyrosyl center and a surface residue at the interface between large and small subunits was suggested (28). In light of this, the allosteric conformation of the large subunit might influence exposure of the radical in the holoenzyme. A thorough in vitro study of this enigma awaits the availability of purified enzyme subunits from cloned sources.

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