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Role of the M2 Subunit of Ribonucleotide Reductase in Regulation by Hydroxyurea of the Activity of the Anti-HIV-1 Agent 2′,3′-Dideoxyinosine*

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ABSTRACT. The ribonucleotide reductase inhibitor hydroxyurea exhibits potent synergism, even at low, non-cytotoxic concentrations, with the anti-HIV-1 dideoxynucleoside 2′,3′-dideoxyinosine, bringing about failure of HIV DNA synthesis and, thus, of HIV replication. To elucidate the incompletely defined role of hydroxyurea in the hydroxyurea/dideoxyinosine interaction and, in particular, to identify the reasons for the unusual selective inhibitory action of the combination on retroviral rather than on cellular DNA synthesis, we prepared specific cDNA probes to determine the effects of low-level hydroxyurea on mammalian cell ribonucleotide reductase M1 and M2 subunit mRNA, while simultaneously quantitating the effects of the drug on cell cycle and on deoxynucleoside triphosphate pools. While dTTP, dCTP, and dGTP pools changed little or even increased in the presence of low-level hydroxyurea, there took place a rapid and specific inhibition of M2-subunit-catalyzed generation of dATP, with consequent slowing of cellular DNA synthesis and prolongation of S phase. However, the latter effect, in turn, resulted in increased M2 subunit mRNA transcription (a process blocked in G_0/G_1-phase cells, with full-length functional M2 transcripts being generated only during S phase) and, hence, in a return to normal levels of dATP and to a normal rate of cellular DNA synthesis. Because of this self-regulating mechanism, hydroxyurea-induced host-cell toxicity was obviated under conditions where HIV DNA synthesis, a process sensitive to both dATP depletion and the chain-terminating properties of the other inhibitory component of the combination (ddATP derived from dideoxyinosine), was unable to recover. BIOCHEM PHARMACOL **56**;1:105–112, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. dideoxynucleosides; combination therapy; antiviral drugs; drug resistance; HIV-1 mutants; HIV-1 replication

Of the metabolites required from the host cell for successful replication of HIV-1¶, the most essential, and thus the most susceptible to pharmacological attack, would appear to be host-cell dNTPs. At any given time, the dNTPs of mammalian cells are far less than 1% of the amount needed for synthesis of the entire genome, so that an interruption of the continuous supply of any one of them can bring a halt to DNA replication, not only for the host cell but even more particularly for the virus, whose capacity for independent dNTP synthesis is entirely lacking. We and other groups have reported recently that exposure to low, non-cytotoxic levels of the ribonucleotide reductase inhibitor HU, by selectively limiting the supply of the essential host-cell deoxynucleotide dATP, markedly enhances the pharmacological activity of the anti-HIV agent ddI [1–3]. In initial clinical studies by Vila and coworkers [4, 5], 1-year treatment with low-dose HU and ddI produced a large reduction of HIV RNA (in some patients, to non-quantifiable levels), not only in the plasma, but also in lymph nodes, the major site of viral replication. Similar clinical results have been reported recently by Lori and coworkers [6, **, ††], who, in addition, demonstrated that

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¶ *Abbreviations:* ddATP, 2′,3′-dideoxyATP; ddI, 2′,3′-dideoxyinosine; ddNs, 2′3′-dideoxynucleosides; dNTPs, 2′-deoxynucleoside-5′-triphosphates; HIV-1, human immunodeficiency virus type 1; HU, hydroxyurea; RT, reverse transcriptase; and PCR, polymerase chain reaction.

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** Lori F, Malykh AG, Foli A, Maserati R, De Antoni A, Wainberg MA and Lisziewicz J, Overcoming drug resistance to HIV-1 by the combination of cell and virus targeting. *Abstracts of the 4th Conference on Retroviruses and Opportunistic Infections*, Washington, DC, January 1997; p. 173.

†† De Antoni A, Foli A, Lisziewicz J and Lori F, Analysis of mutations of HIV-1 reverse transcriptase after therapy with ddI plus hydroxyurea. *Abstracts of the 4th Conference on Retroviruses and Opportunistic Infections*, Washington, DC, January 1997; p. 174.

the HU/ddI combination retained its activity against ddI-resistant mutants emerging during therapy with the combination. However, the mechanism of action of low-dose HU in the HU/ddI combination has not been fully defined. At high (cytotoxic) concentrations, such as those used in cancer chemotherapy and in the treatment of hemoglobinopathies, single-agent HU is known to quench a catalytically active tyrosyl free radical of the non-heme iron-containing M2 subunit of ribonucleotide reductase, with resultant dNTP depletion and cell cycle arrest in early S phase [7, 8]. However, since host-cell cytotoxicity is minimal or absent with the low-dose HU/ddI combination, Malley and co-workers [9] have suggested the possibility of alternative mechanisms for low-dose HU, e.g. interaction of HU with metal-ion binding proteins of the virus itself (such as the zinc finger sequences of the HIV-1 P7 nucleocapsid protein), rather than the inactivation of tyrosyl radicals of the host cell ribonucleotide reductase enzyme. To examine the role of low-dose HU more closely, we have prepared specific cDNA probes by RT-coupled PCR for the direct quantitation of ribonucleotide reductase M1 and M2 subunit mRNA in human cells exposed to the HU concentrations utilized in the low-dose HU/ddI protocol, while defining the temporal relationships between host-cell ribonucleotide reductase mRNA generation, dNTP synthesis, and cell-cycle progression.

MATERIALS AND METHODS

Materials

HU and phytohemagglutinin were obtained from the Sigma Chemical Co. ddI was supplied by the Pharmaceutical Resources Branch, Division of Cancer Treatment, NCI. [2′,3′-^{3}H]ddI (38 Ci/mmol) and [2-^{14}C]thymidine (55 mCi/mmol) were purchased from Moravek Biochemicals. Recombinant interleukin-2 was purchased from R & D Systems. Nucleoside and nucleotide standards were purchased from Sigma or from Pharmacia. The restriction enzymes utilized were purchased from Promega.

Cells

CCRF-CEM cells (obtained from the American Type Culture Collection) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air/5% CO_2. Cell growth experiments were carried out by plating uninfected CEM cells at an initial density of 2×10^4 cells/mL in the presence and absence of the drugs under study (HU, ddI), and harvesting 10-mL aliquots for counting at 24, 48, and 72 hr. Viability was assessed by trypan blue exclusion. Cell replication experiments were repeated three times. For assessment of the potentiation of the anti-HIV activity of ddI by HU, 2×10^4 CEM cells were incubated with ddI concentrations ranging from 0.2 to 2.0 μM in the presence or absence of 50 μM of HU. At 12 hr, HIV-1_{LAI} was added at a dosage of 1000 tissue culture 50% infectious doses/2×10^4 cells. The virus-containing medium was harvested at day 7, and HIV p24 antigen was determined by radioimmunoassay. Potentiation experiments were carried out three times, with quadruplicate determinations in each experiment.

Quantitation of Ribonucleotide Reductase M1 and M2 Subunit mRNA

Total RNA was isolated from logarithmically growing CEM cells by a phenol/chloroform extraction method [10]. RNA was electrophoresed on a 5% formaldehyde/1% agarose gel and then blotted onto a Hybond-N membrane (Amersham). The full-length cDNA probes of human ribonucleotide reductase M1 and M2 subunits were synthesized by RT-coupled PCR as previously described [10, 11]. The primers for synthesis of the M2 probe were the 5′ primer from positions 189 to 211, containing the AUG site (5′-ATCCGGATCCACTATGCTCTCCCTCCGTGT 3′), and the 3′ primer from positions 1346 to 1368 including the UAA site (5′-GCTTAAGCTTATTTA GAAGTCAGCATCCAAG-3′). The primers for the M1 probe were a 5′ primer from positions 188 to 207, including the AUG site (5′-GCTCGAGCTCATGCATGTGAT CAAGCGA-3′) and a 3′ primer from positions 2549 to 2570 containing the UGA site (5′-GCAGCTGCAGCT CAGGATCCACACATCA-3′). The PCR products were ligated into TA cloning vectors (Invitrogen) [10] and amplified by *Escherichia coli*. The restriction enzymes *Pst*I and *Sac*I were used to remove M1 from the vector, and the restriction enzymes *Hin*dIII and *Bam*HI were used for M2. After enzyme digestion, full-length cDNA probes were verified by Miniprep gel analysis and ^{32}P-labeled for probing the RNA blots. To obtain equal intensity, the autoradiographs of the M2 and M1 subunits were exposed for 24 and 6 hr, respectively. β-Actin sequences were amplified from the same RNA extracts to normalize the amount of RNA used.

Quantitation of dNTP Pools

The method used was a modification of the highly sensitive enzyme assay described by Sherman and Fyfe [12]. Sequenase Version 2.0 DNA polymerase was obtained from the United States Biochemical Corp. All oligonucleotides used as template primers were purchased from Genosys Biotechnologies, Inc. The universal primer was mixed with and annealed to each template (1:1 ratio) by heating at 65° for 10 min and slowly cooling to room temperature. The sequences of the oligonucleotides were as follows:

TEMPLATES. For dCTP determination, 5′-TTTGTTTGT TTGTTTGTTTGGGCGGTGGAGGCGG-3′; for dTTP determination, 5′TTATTATTATTATTATTATTAGG CGGTGGAGGCGG-3′; for dGTP determination, 5′-TT

TCTTTCTTTCTTTCTTTCGGCGGTGGAGGCGG-3′; for dATP determination, 5′-AAATAAATAAATAAAT AAATGGCGGTGGAGGCGG-3′.

UNIVERSAL PRIMER. 5′-CCGCCTCCACCGCC-3′.

PREPARATION OF CELL EXTRACTS. CEM cells, maintained as described above, were washed twice with cold PBS, and subjected to extraction with 60% methanol. The extracts were heated for 5 min at 95° and kept frozen at −70° until assay.

DETERMINATION OF dNTPs. The reaction mixture for Sequenase 2.0 was prepared to contain 0.05 U Sequenase, 50 mM of Tris-HCl, pH 7.5, 10 mM of $MgCl_2$, 5 mM of dithiothreitol, 0.25 μM of template primer, 2.5 μM of [^{3}H]dATP (15 Ci/mmol, for dCTP, dTTP, and dGTP determinations) or 2.5 μM of [^{3}H]dTTP (15 Ci/mmol for dATP determination). Then extract from approximately 1×10^5 cells (5 μL) was added to 45 μL of the reaction mixture to bring the total volume to 50 μL. The reaction was carried out at 26° for 20 min, followed by spotting 40 μL of the reaction mixture onto Whatman DE81 paper. The filters were washed extensively three times in 5% Na_2HPO_4 (10 min per wash), rinsed with distilled water and then with 95% ethanol, and dried. The radioactivity on the filters was determined by liquid scintillation counting.

Cell Cycle Analyses

CEM cells (5×10^6) were washed with PBS, fixed with ice-cold 70% ethanol, and stored at 4° until further treatment. Fixed cells were incubated with ribonuclease A (DNase-free, 1 mg/mL) and propidium iodide (0.05 mg/mL) at 37° for 30 min. Samples (10^4 cells per assay) were analyzed using a Coulter Epics (XL) flow cytometer (Coulter Corp.) with a 488-nm argon laser running at 15 mW. Doublet exclusion analysis was performed by gating on a linear-peak signal histogram.

Thymidine Incorporation Studies

The time dependence of the effect of 0.1 mM of HU on the rate of DNA synthesis was assessed by thymidine incorporation assays. CEM cells (5×10^5/mL for 0, 0.2, 0.5, 1, 8, and 24-hr time points, and 2.5×10^5/mL for 35 and 48 hr) were exposed to [2-^{14}C]thymidine (55 mCi/mmol) in the presence and absence of 0.1 mM of HU. After incubation, the cells were washed three times with ice-cold PBS, and the acid-soluble fraction was extracted twice with 0.5 N perchloric acid. ^{14}C-Radioactivity in the perchloric acid-insoluble fraction was determined by liquid scintillation counting. Results are expressed as picomoles thymidine incorporated per 10^6 cells per hour.

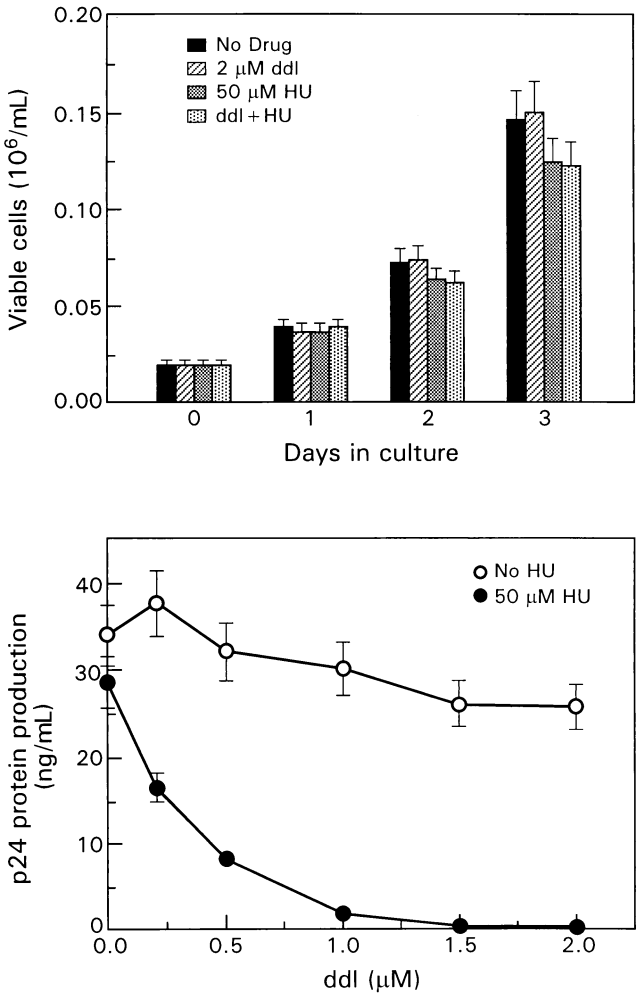

FIG. 1. Effects of HU and ddI on HIV-infected and uninfected CEM cells. Upper panel: CEM cells plated at an initial density of 2×10^4 cells/mL were incubated with 2 μM of ddI and/or 50 μM of HU. Cell counts were performed every 24 hr for 3 days. Results shown are the means ± SD of 3 independent experiments. Lower panel: CEM cells (2×10^4 cells/assay) were incubated with ddI concentrations ranging from 0.2 to 2.0 μM, with or without the addition of HU, 50 μM. At 12 hr, HIV_{LAI} was added (1000 tissue culture 50% infectious doses/2×10^4 cells). On day 7, p24 antigen was determined by radioimmunoassay. Results shown are the means ± SD of 3 independent experiments, each carried out in quadruplicate.

RESULTS

Effects of ddI and of Low-Dose HU on CEM Cell Replication Rate

ddI (2 μM) had no measurable effect on CEM cell replication when assessed at 24, 48, and 72 hr. No change in replication rate was seen with low-dose HU (50 μM) over 24 hr, but slight (18%) inhibition was noted when cell growth was measured over 72 hr (Fig. 1, upper panel). A similar effect of low-dose HU on CEM cell growth rate has been reported by Karlsson and coworkers [13]. No additional inhibition of replication rate was noted with the ddI/HU combination over that seen with HU alone (Fig. 1, upper panel).

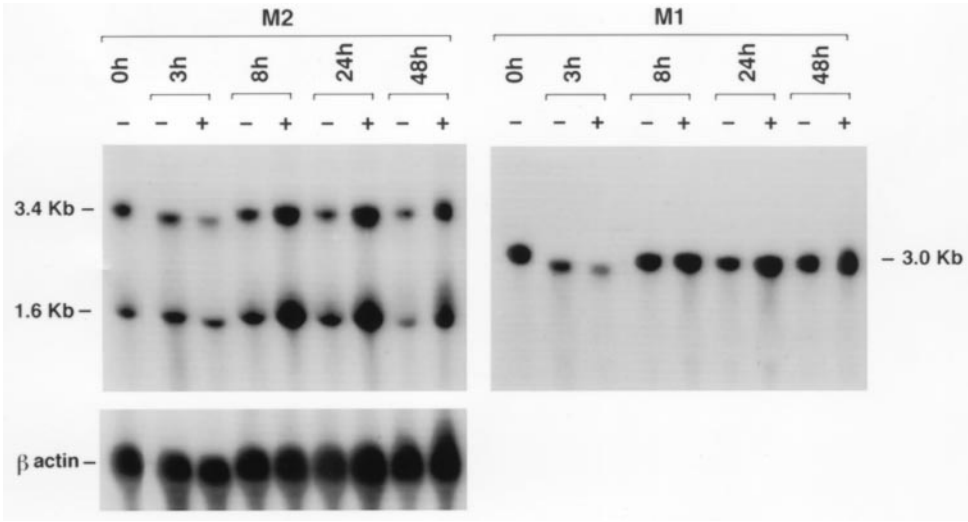

FIG. 2. Northern blot analyses of ribonucleotide reductase M1 and M2 subunit mRNA in CEM cells. Total cellular RNA (20 μg per lane) was extracted from CEM cells in logarithmic growth phase in the presence or absence of 0.1 mM of HU for 0, 3, 8, 24, and 48 hr. For preparation of M1 and M2 cDNA probes and subsequent methodology, see Materials and Methods. Representative analyses for three experiments are shown, with blot densitometry values varying < 5% between experiments. β-Actin cDNA probes were used to quantitate the relative amounts of mRNAs in each preparation. Key: (M1) M1 subunit mRNA; (M2) M2 subunit mRNA; (−) in the absence of HU; and (+) in the presence of 0.1 mM of HU.

Effects of ddI/HU Combinations on HIV-1 Replication in CEM cells

When assessed by p24 protein production at 7 days, ddI alone showed only slight inhibition of HIV-1 replication in CEM cells over the concentration range examined (9 and 21% at ddI concentrations of 1.0 and 2.0 μM, respectively; Fig. 1, lower panel). In the presence of 50 μM of HU, however, markedly enhanced inhibition by ddI was observed (92% inhibition at 1.0 μM of ddI and complete inhibition at 2.0 μM of ddI). The 50% inhibition concentration for ddI in the presence of 50 μM of HU was 0.3 μM (Fig. 1, lower panel).

Effects of Low-Dose HU on Ribonucleotide Reductase M1 and M2 Subunit mRNA

CEM cells growing in logarithmic phase were exposed to low-dose (0.1 mM) HU and harvested at 0, 3, 8, 24, and 48 hr. Total cellular RNA was extracted and analyzed by northern blots, using as probes PCR products with the full-length cDNA sequences of human ribonucleotide reductase M1 and M2 subunits. As shown in Fig. 2, at the earliest time point (3 hr), HU exposure resulted in a slight decline in both 3.4 and 1.6 kb M2 mRNAs (to levels of 0.76 and 0.78-fold, respectively, of the levels of the no-drug controls, as determined by densitometry analysis). Enhanced generation of both M2 mRNAs commenced between 3 and 8 hr of HU exposure, with 2- to 3-fold increases being noted at 8, 24, and 48 hr (maximal at 24 hr). A much slighter increase in M1 mRNA was also noted at the three later time points. No change was observable in β-actin expression throughout the period of HU exposure.

Effects of Low-Dose HU on dNTP Pools and on DNA Synthesis

Because ribonucleotide reductase catalytic activity is rate-limited by the M2 subunit, we determined next the temporal relationship between changes in M2 mRNA levels and dNTP pools and also cellular DNA synthesis, upon exposure to low-dose HU. As shown in Fig. 3, a striking decline in the dATP pool was noted at the earliest time point measured, with the dATP pool continuing to decrease until reaching a minimum (28% of control) at 8 hr. Substantial recovery (to 58% of control) was noted by 16 hr, and the recovery continued until the dATP pool returned to control levels at about 35 hr. In contrast, very little change was noted in dGTP, dCTP, and dTTP pools over the first 5 hr. Over the 5- to 12-hr time period, a slight decline was noted in all three of the latter dNTPs, followed by a recovery to normal or even increased levels during the remaining period of HU exposure. The rate of DNA synthesis (as assessed by thymidine incorporation) showed a striking decrease at the earliest measurable time point (0.2 hr), reaching a nadir of about 15% of control over the 1- to 6-hr time period (Fig. 4). A rapid recovery to or slightly above control levels occurred over the time period 8–13 hr, coincident with the recovery in dATP pools.

Effects of Low-Dose HU on Cell Cycle

HU, at higher dose levels, is known to be an effective cell synchronizing agent, with the cell cycle being blocked at the G_1/S boundary [8]. To determine the effects of low-dose HU on progression through the cell cycle, and assess the temporal relationship of cell-cycle changes to the changes

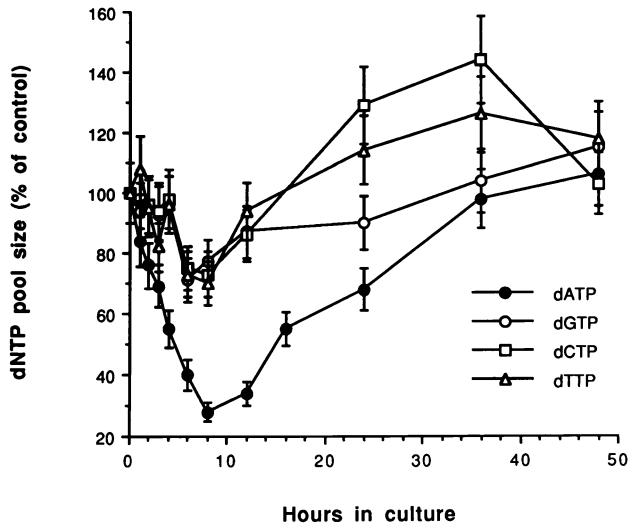

FIG. 3. Reversible inhibition of dNTP pool size by low-level HU. CEM cells (10^7 per assay) were incubated at 37° in the presence and absence of 0.1 mM of HU. Cells were harvested at the indicated times, and intracellular dNTP pools were determined as described in Materials and Methods. Three independent pool size determinations were carried out; results shown are the averages of duplicate analyses from a typical experiment. The values of no-drug controls at 0 hr for dATP, dGTP, dCTP, and dTTP were 18, 16, 26, and 31 pmol/10^6 cells, respectively.

noted in ribonucleotide reductase mRNA expression and in dATP and cellular DNA synthesis, we carried out flow cytometry studies on an asynchronously replicating population of CEM cells at the same low HU level as used in the earlier studies. As shown in Fig. 5, the first effects noted (0–12 hr) were a substantial drop (50%) in cells in G_2/M phase, with a slight fall in the percentage of cells in S phase and a corresponding slight increase in cells in G_1. At 8–12 hr, however, a sharp 2.3-fold increase in S-phase cells commenced (from 35 to 81% at 24 hr) together with a parallel fall in the percentage of cells in G_1 (from 52 to 11%). Over the following 24 hr, a reversal of these HU-induced changes was noted, with both S and G_1-phase cells returning to their pretreatment control distribution percentages.

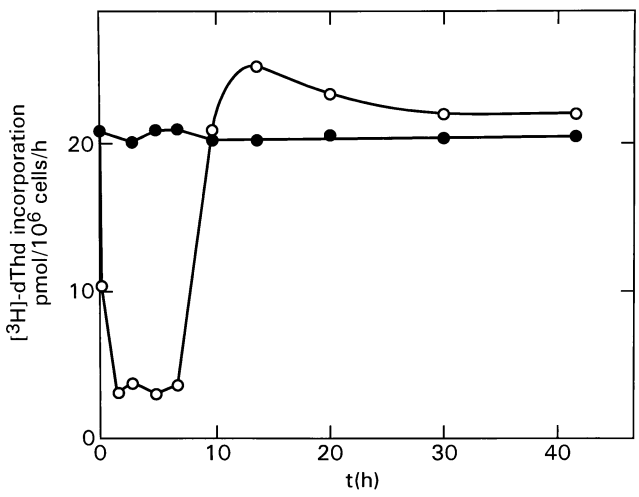

FIG. 4. Time dependence of inhibition of thymidine incorporation. CEM cells (5×10^5/mL for 0, 0.2, 0.5, 1, 8, and 24 hr time points, and 2.5×10^5/mL for 36 and 48 hr time points) were exposed to [^{14}C]thymidine (55 mCi/mmol) in the presence (○) and absence (●) of 0.1 mM of HU. Thymidine incorporation was determined as described in Materials and Methods. Results shown are the averages of duplicate determinations.

DISCUSSION

These observations in CEM cells with low-dose HU, considered together with our earlier studies in PBM cells [1, 3], appear compatible with the following interpretation, outlined schematically in Fig. 6. Paralleling the HU-induced decrease in dATP pools is an abrupt decline in the rate of cellular DNA synthesis, confirming the extreme sensitivity of the latter process previously noted by Reichard and coworkers [14] to even a brief interruption in the supply of this essential deoxynucleotide. The unusual susceptibility of dATP pools to depletion by HU (relative to other dNTPs) may be a consequence of the rapid removal and cleavage of its potential endogenous precursor deoxynucleoside, deoxyadenosine, by the successive action of adenosine deaminase and purine nucleoside phosphorylase, together with the low substrate activity of deoxyadenosine for direct phosphorylation by deoxyadenosine/deoxycytidine kinase [15]; the result is the primary reliance for dATP supply on *de novo* synthesis (via the HU-sensitive enzyme ribonucleotide reductase) rather than on salvage pathways.

Following these initial changes is a decrease in cells in G_2/M phase of the cell cycle and an increase in cells in G_1 phase, indicating that cells are exiting G_2/M but are not being replaced at an equivalent rate from S phase. With cells retaining the ability to progress through G_2/M and G_1 but unable to complete DNA synthesis in S phase, the percentage of cells in the latter increases steadily, from 38% at 12 hr to 81% at 24 hr ("partial synchronization").

The compensatory increase in ribonucleotide reductase M2 mRNA is not seen at the earliest time point examined (3 hr), but develops in parallel with the later accumulation of cells in S phase, becoming readily detectable at 8 hr and maximal at 24 hr. The temporal correlation between accumulation of cells in S phase and the increase in M2 mRNA transcripts is compatible with recent observations by Björklund and coworkers [16, 17]. These authors demonstrated that while short incomplete ribonucleotide reductase M2 mRNA transcripts are synthesized during G_1 phase, a transcriptional block prevents the synthesis of full-length functional transcripts during G_1, with the block being released only as cells enter S phase. Thus, in the present study, with the resulting increase in functional ribonucleotide reductase M2 mRNA and consequently in M2 subunit catalytic activity during the prolonged S phase, pools of dATP are restored and cellular DNA synthesis returns to normal levels at 15–20 hr *even in the continued*

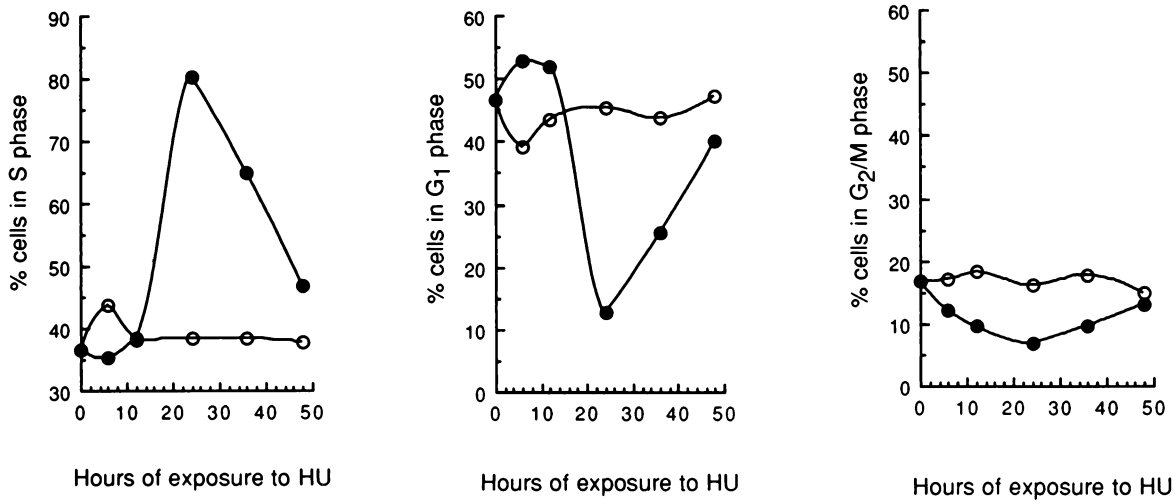

FIG. 5. Effects of low-dose HU on CEM cell cycle. CEM cells were exposed to 0.1 mM of HU for the indicated time periods. The percentages of cells in S phase, (left panel), G_1 (center panel), and G_2/M (right panel) were determined by flow cytometric analysis as described in Materials and Methods. Three separate cell cycle experiments were carried out; results shown are the averages of duplicate values from a single experiment. Key: (○) no-drug controls; and (●) plus 0.1 mM of HU.

presence of low-level HU, thus obviating irreversible cytotoxicity. These studies, therefore, while not ruling out other contributory mechanisms for the action of HU in the HU/ddI combination, support the contention that the effects consequent on HU exposure can be explained adequately on the basis of known properties of mammalian ribonucleotide reductase.

With reference to the other component of the combination (ddATP generated intracellularly from ddI), its synthesis proceeds through routes wholly independent of dATP generation [18], and thus remains unaffected by changes in ribonucleotide reductase activity. As we have demonstrated previously, ddATP formation proceeds even in "resting" or "G_0" lymphocytes [19] because the 5′-nucleotidase responsible for the initial phosphorylation of ddI is cell-cycle independent. Furthermore, as we previously showed in MOLT-4, CEM, and PBM cells [20, 21], the persistence of ddATP, once generated, is extremely long, with a decay $t_{1/2} > 40$ hr, so that the drug persists intracellularly at pharmacologically active levels long after ddI either is removed or diffuses away from the extracellular medium. In this respect, ddI differs from other 2′,3′-dideoxynucleoside anti-HIV agents (3′-azido-2′,3′-dideoxythymidine, 2′,3′-dideoxycytidine), whose intracellular 5′-triphosphates have relatively brief decay half-times (3–6 hr) [21]. This unusual property of intracellular ddATP would appear to be responsible, in part, for the difference in HU potentiation between ddI and other anti-HIV dideoxynucleosides noted by Malley and coworkers [22].

Two interrelated questions arise from these studies: 1)

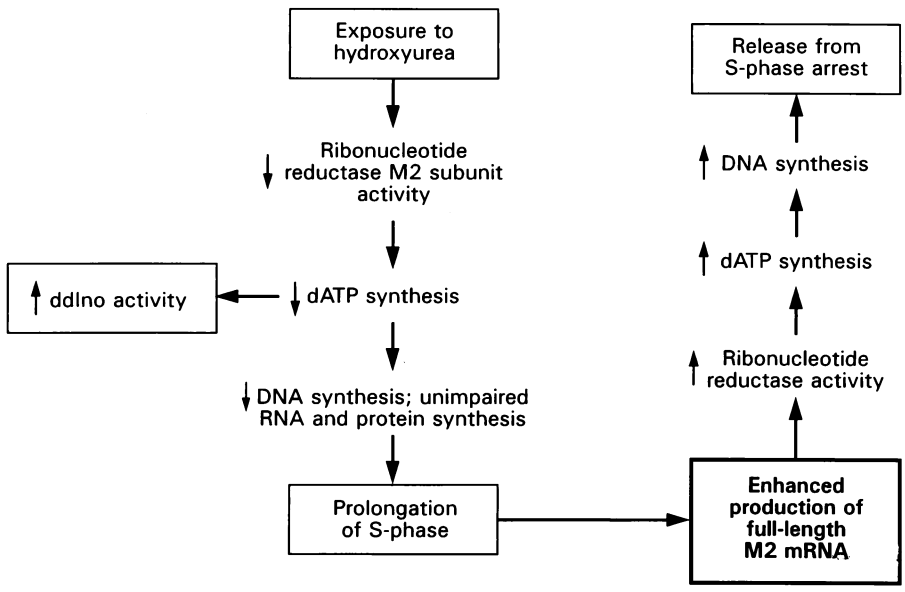

FIG. 6. Schematic depiction of the proposed roles of the ribonucleotide reductase M2 subunit and of dATP pool-size in reversible S-phase arrest and release induced by exposure to low-dose HU.

why is the HU/ddI combination, although highly effective in inhibiting the replication of HIV DNA, not equally effective in inhibiting chromosomal DNA synthesis; and 2) because dATP levels return to normal with the recovery of ribonucleotide reductase activity, why does increased dATP not reverse the inhibition of HIV DNA synthesis as well as that of host cell genomic DNA synthesis?

The reasons for these differences in the susceptibility of host cell and HIV DNA synthesis most probably arise from the differences in the susceptibility to ddATP of the polymerases responsible for these processes, i.e. DNA polymerases α and δ for chromosomal DNA replication, and RT for HIV DNA replication. It has long been known that, although the mitochondrial DNA polymerase γ is susceptible to inhibition by a variety of ddNTPs [23, 24], DNA polymerase α retains significant ability to discriminate between physiological deoxynucleotides and non-physiological dideoxynucleotides; the viral polymerase, however, with its decreased replication fidelity and its lack of effective proofreading capacity, is much more susceptible to the inhibitory and the chain-terminating effects of ddATP. In addition, without associated 3′-5′-exonuclease activity, further progression of HIV DNA synthesis is effectively blocked once chain-termination by ddATP occurs [25]; thus, the return of dATP to normal levels cannot bring about the resumption of HIV DNA chain extension. Chromosomal DNA synthesis, however, being insensitive to the inhibitory effects of ddATP, is only slowed by the dNTP imbalance resulting from ribonucleotide reductase inhibition, and is able to resume with the restoration of normal dATP levels.

A final implication of these studies would appear to be that there is no fundamental reason why a similar strategy of dNTP depletion cannot be applied to potentiate the anti-HIV effects of other ddNTPs. While HU and other ribonucleotide reductase inhibitors, when utilized at low concentrations, are primarily effective in selectively reducing dATP levels, and thus in potentiating inosine and adenosine-based ddNs, other pharmacological agents are capable of selectively reducing dTTP, dCTP, or dGTP, and thus of potentiating such agents as 2′,3′-dideoxythymidine-2′,3′-ene (D4T), zidovudine (AZT), 2′,3′-dideoxycytidine, and carbovir. Such combinations have yet to be explored beyond the *in vitro* level.

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