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Determination of Deoxyribonucleoside Triphosphate Pool Sizes in Ribonucleotide Reductase cDNA Transfected Human KB Cells

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ABSTRACT. Ribonucleotide reductase (RR) is a rate-limiting enzyme in DNA synthesis, which is responsible for controlling deoxyribonucleoside triphosphate (dNTP) pool size. It has been shown that transfection of RR M2 cDNA in human KB cells (M2-D clone) results in overexpression for the M2 subunit and resistance to hydroxyurea (HU). In this study, dNTP pool assays were performed to measure the pool sizes in six cell lines: two controls, three transfectants, and drug-induced HU-resistant (HUR) cells. Total dNTP levels among the six cell lines rose in the following order: KB wild-type, KB vector-only transfectant, M1 cDNA transfectant, M2 cDNA transfectant, M1/M2 cDNA transfectant, and HU-induced resistant clone. The dCTP levels of the cells mimicked the total dNTP pools on a smaller scale. The significant increases in the dCTP pool sizes of the M2-D, X-D, and HUR clones were proportional to their respective increases in RR activity. Relative to all other transfectants, the M1-D clone demonstrated lower dCTP levels but increased dATP pools. The M1-D clone demonstrated a significant resistance to dNTP inhibition of RR activity compared with the control KB wild-type cells. In contrast, a profound inhibition of dCTP and a decreased sensitivity to dATP inhibition was observed in M2-D, X-D, and HUR clones. In summary, M2 cDNA transfectants and HUR clones had increased RR activity as well as expanded dNTP pools, particularly dCTP, when compared with wild-type KB cells. These data provide evidence for the intertwined relationship between RR activity and dNTP pools. BIOCHEM PHARMACOL **55**;10:1657–1665, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. ribonucleotide reductase; deoxyribonucleoside triphosphate pools; hydroxyurea; drug resistance

The balance between dNTP† pools directly affects cell function and proliferation [1]. Lowered or perturbed dNTP pools may result in mutagenesis or chromosomal instability [2]. However, maintenance of dNTP pools depends on an extremely complex system of over 60 different enzymatic reactions [3]. RR, a highly regulated enzyme, is S-phase specific and controls the rate-limiting step in the DNA synthesis pathway [4]. With one exception, RR catalyzes the *de novo* conversion of all common ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates [5]. The exception is dTTP, which results from the reduction of either UDP or CDP, with the introduction of a methyl group at the monophosphate level. The dNTPs manufactured by RR either can enter the nucleus to become substrates for DNA polymerase or can remain in the cytoplasm and act as positive or negative effectors in the regulation of RR substrate specificity. dNTPs can also be formed by a salvage pathway via phosphorylation of the corresponding deoxyribonucleosides when these are available [6].

The enzyme RR consists of two components: the M1 subunit, a 170,000 dimer that contains the effector binding site necessary for allosteric regulation, and the M2 subunit, a 88,000 dimer that contains a tyrosine free radical and a nonheme iron [7]. A prominent chemotherapeutic agent, HU, specifically inhibits RR by binding to the protein M2, quenching the tyrosine free radical, and consequently halting RR enzyme activity [8].

It has been well documented that elevations in M2 mRNA and protein levels exhibit close correlations with HU resistance and changes in DNA synthesis [9–12]. In previous papers, we have discussed two findings: (a) RR is overproduced in clones of human HU-resistant cells selectively established from stepwise increases of HU [13], and (b) overexpression of wild-type RR M2 cDNA efficiently increases enzymatic activity and resistance to HU [14]. In this study, we cultured human KB transfectants that were transfected with RR M1, M2, and M1/M2 cDNA fused to a β-actin promoter. After selection of stable transfected cell lines, we examined the sizes of dNTP pools, RR mRNA

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† *Abbreviations:* dNTP, deoxyribonucleoside triphosphate; DTT, dithiothreitol; HU, hydroxyurea; HUR, HU resistant; and RR, ribonucleotide reductase.

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expression, and RR activity in these cells to determine whether RR activity was correlated to dNTP pool size changes.

MATERIALS AND METHODS

Cells

Human oropharyngeal carcinoma KB cells (American Type Culture Collection) were cultured with 5% carbon dioxide at 37° on plastic tissue culture plates in RPMI 1640 medium supplemented with 10% fetal bovine serum. A clone of HUR cells (a gift from Dr. Y. C. Cheng) was sequentially selected in a stepwise manner in the presence of 1 mM HU. M1-D, M2-D, and X-D cells were transfected with M1 cDNA, M2 cDNA, and M1/M2 cDNA, respectively, which were then cultured in the presence of 10% fetal bovine serum [14].

Templates, Primers, Radioactive Materials, and Ion Exchange Papers

All templates and primers were synthesized at the City of Hope facility. [^{3}H]dATP and [^{3}H]dTTP were purchased from Moravek Biochemicals. DE81 ion exchange paper was purchased from Whatman, Ltd.

Harvesting Cells

Cells were washed with PBS and detached with 0.2 mL of trypsin. Cells were transferred into a 15-mL tube and washed again with PBS. A 100-μL sample was dyed with Trypan Blue and counted under a microscope. The remainder of the solution was centrifuged at 1000 rpm for 5 min, transferred into a 1.5-mL Eppendorf tube, and centrifuged for 5–7 sec.

Isolating dNTPs

One hundred microliters of 15% trichloroacetic acid was added to the cell pellet. The solution was mixed and kept on ice for 10 min. Then it was centrifuged for 5 more minutes, and the supernatant was saved. dNTPs were extracted with two 50-μL aliquots of freon/trioctylamine (55%:45%). After each centrifugation, the supernatant was saved. Two 5-μL aliquots (one for duplicate) of each sample were used to check the dATP, dCTP, dGTP, and dTTP concentrations. The remainder was stored at −80° for further assays.

dNTP Pool Assay

This assay was conducted according to the method of Sherman and Fyfe [15]. The total reaction volume was 50 μL. The reaction mixture contained 50 mM of Tris-HCl (pH 7.5), 10 mM of $MgCl_2$, 5 mM of DTT, 0.25 μM of template/primer, 1.25 μM of [^{3}H]dATP (for dCTP, dGTP, and dTTP assay) or [^{3}H]dTTP (for dATP assay), and 0.2 units of Sequenase (2.0). The mixture was allowed to react at room temperature for 20 min. After incubation, 40-μL aliquots were removed and spotted onto circular (diameter 2.4 cm) Whatman DE81 ion exchange papers. The papers were dried, washed (3 × 10 min) with 5% Na_2HPO_4, and rinsed once with distilled water and once more with 95% ethanol. After each paper was dried and deposited in a small test tube, 5 mL of Ecolume was added to each tube. Tritium-labeled dNTPs were counted by a Liquid Scintillation Counter. Standard samples were 0.25, 0.5, and 1.0 pmol.

RNA and Northern Blot Analysis

Total RNA was extracted from logarithmically growing cells as described previously [13]. RNA was electrophoresed in a formaldehyde-agarose gel and blotted onto Hybond-N membrane. Radioactive probes were prepared as described previously. Hybridizations were performed under stringent conditions.

RR Assay

RR activity was measured utilizing the CDP assay method of Steeper and Steuart [16]. Cells were washed with cold PBS and detached by trypsin and a cell scraper. Cells were transferred into a 15-mL tube and pelleted by centrifugation at 300 *g* for 10 min at 4°. The pellets were washed again with PBS. One volume of low salt homogenization buffer (10 mM of HEPES, pH 7.2, with 2 mM of DTT) was added to the cell pellets. The cell suspension was passed through a No. 27G½ needle 20 times on ice. After homogenization, another 1 vol. of high salt buffer (1 M of HEPES, pH 7.2, with 2 mM of DTT) was added. The cell suspension was passed through a No. 27G½ syringe needle 20 times on ice. Cell debris were removed by centrifugation at 16,000 *g* at 4° for 20 min. The supernatant was passed through a Sephadex G25 spin column, which pre-equilibrates with buffer (50 mM of HEPES, pH 7.2, 2 mM of DTT) to remove endogenous nucleotides. Protein concentration was measured by the Bio-Rad protein assay. The reaction mixture contained the following in a final volume of 0.15 mL: [^{14}C]CDP (0.02 μCi; 0.15 mM), HEPES (pH 7.2) (50 mM), DTT (6 mM), magnesium acetate (4 mM), ATP (2 mM), CDP (0.05 mM), and a specific amount of cell extract. The incubation time for the reaction was 20 min. The amount of dCDP formed was dephosphorylated by phosphodiesterase. Then C and dC were separated with a C_{18} ion exchange column by HPLC. The reaction was linear during the process. For dNTP inhibition study, the cell extract was mixed with a designated concentration of dNTP. After a 30-min preincubation under 37°, the RR reaction mixture was added, and then the above-mentioned procedure for RR assay was performed.

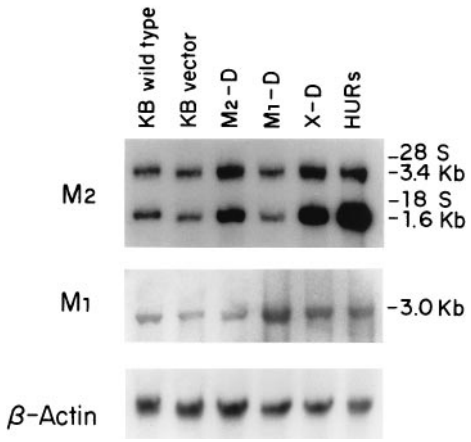

FIG. 1. Northern blot analysis of M2 and M1 mRNA expression in transfectants, KB wild-type and HUR cells. Thirty micrograms of total RNA was isolated from KB wild-type and HUR cells and from each of the transfectants. The positions of 28S and 18S are shown. A β-actin probe was used to quantify the relative amounts of mRNA in each preparation.

RESULTS

RR mRNA Expression, RR Enzyme Activity, and HU Resistance in Transfectants

Northern blots probed with M2 and M1 cDNA showed that various clones expressed different levels of mRNA in comparison with KB wild-type values (Fig. 1). Probed with M2 cDNA, two bands at 3.4 and 1.6 kb mRNA were detected. The vector-transfected clone exhibited the same level of M2 mRNA as KB wild-type cells. M2-D demonstrated an approximately six-fold higher level of M2 mRNA expression than the wild-type KB and the vector transfectant. X-D demonstrated a three-fold higher level of M2 mRNA than control clones. The M1-D clone revealed no increase in the expression of M2 mRNA. HUR cells, however, expressed an approximately 10-fold higher level of M2 mRNA than KB wild-type cells. Probed with M1 cDNA, only the M1-D clone and the X-D clone demonstrated an approximately 3-fold higher level of M1 mRNA expression than wild-type KB cells, HUR cells, and the M2-D clone.

To determine the RR activity level of each of the transfectants, cell extracts were prepared and RR activity levels were determined utilizing [^{14}C]CDP as a substrate. The results are summarized in Table 1. The vector-transfectant, KB-V, demonstrated the same level of RR activity as the KB wild-type cells. In the X-D and M2-D clones, RR activity increased to three-fold that of the KB wild-type cells. The M1-D clone demonstrated no difference from the control. The HUR cells exhibited a six-fold elevation of RR activity compared with the KB wild-type cells.

The sensitivity of KB wild-type cells, HUR cells, and transfectants to HU is also summarized in Table 1. The IC_{50} values of HU for KB wild-type, HUR, and vector transfectants were 0.3, 4.5 and 0.3 mM, respectively. The IC_{50} values for the M2-D and X-D clones were 3-fold higher than for the KB wild-type cells. The M1-D transfectant demonstrated no difference from the KB wild-type cells. This result is comparable to the increase in RR activity and M2 mRNA expression of each cell line.

dNTP Pools from Transfectants

The total dNTP level of each cell line increased in the following order: KB wild-type = KB-V, M1-D, M2-D, X-D, and HUR (Table 2). In each cell type, the ratio of dTTP production to total dNTP was the highest (0.4–0.6:1). In KB wild-type cells, the concentrations of dATP and dCTP were virtually equal; dTTP concentration was about four-fold greater than dGTP and six-fold greater than either dATP or dCTP. The KB-V clone revealed no significant changes in individual or total pool levels from KB wild-type cells.

The major difference between the M1-D clone and the KB wild-type cells is that in the M1-D clone the total dNTP pool was expanded to 2.5-fold that of the KB wild-type cells (21.9 vs 8.3). This dNTP pool expansion of M1-D clones is due primarily to an increase in dATP levels to 25% of total dNTP pools. In comparison, the dATP levels of KB wild-type and KB-V comprise only 10% of their total dNTP pools. Both the dCTP:dNTP and dGTP:dNTP ratios in the M1-D clones and the KB wild-type cells remained the same. However, the dTTP:dNTP ratio was decreased 20% from the KB wild-type cells. Therefore, the expansion of dNTP pools in the M1-D clone was due primarily to the dATP increase in quantity.

In M2-D and X-D clones, the total dNTP pools were elevated 3- and 5-fold, respectively, in comparison with

TABLE 1. Values for RR activity and the sensitivity of the indicated cell lines to HU

Cells	Specific RR activity[a] (mmol CDP/hr/mg protein)	Ratio	IC_{50}[†] (mM) of HU	Ratio
KB wild-type	1.0	1×	0.31 ± 0.07	1×
KB-V	0.9 ± 0.13	1×	0.29 ± 0.03	1×
X-D	3.3 ± 0.11	3×	1.02 ± 0.06	3×
M2-D	3.13 ± 0.06	3×	0.97 ± 0.11	3×
M1-D	1.17 ± 0.37	1×	0.29 ± 0.01	1×
HUR	6.73 ± 0.06	6.7×	4.5 ± 0.9	15×

*Enzyme activity was determined as described under Materials and Methods. Values are averages ± SD calculated from three separate experiments, each performed in duplicate.
†The IC_{50} values were determined as described in Materials and Methods by using the methylene blue assay. Values are means ± SD, $N = 3$.

TABLE 2. Concentrations of dNTPs calculated in picomoles for every 1 million cells

Cells	dATP	dCTP	dGTP	dTTP	dNTP	Ratio
KB wild-type	0.79 ± 0.27 (0.1)*	0.90 ± 0.58 (0.1)	1.49 ± 0.65 (0.17)	5.16 ± 2.69 (0.63)	8.34 ± 1.05 (1)	1×
KB-V	0.91 ± 0.23 (0.1)	0.76 ± 0.16 (0.1)	1.66 ± 1.24 (0.2)	4.97 ± 1.34 (0.6)	8.28 ± 0.89 (1)	1×
M1-D	4.76 ± 0.44 (0.25)	3.77 ± 1.25 (0.17)	3.65 ± 2.03 (0.17)	9.71 ± 0.26 (0.41)	21.9 ± 0.99 (1)	2.5×
M2-D	2.84 ± 0.37 (0.1)	5.84 ± 2.13 (0.22)	6.09 ± 1.91 (0.23)	12.6 ± 4.57 (0.45)	26.6 ± 2.24 (1)	3×
X-D	3.35 ± 0.20 (0.08)	9.90 ± 0.24 (0.25)	7.84 ± 1.67 (0.21)	18.0 ± 1.54 (0.46)	39.1 ± 0.91 (1)	5×
HUR	5.57 ± 1.78 (0.12)	13.4 ± 6.73 (0.3)	7.16 ± 4.87 (0.15)	19.2 ± 6.14 (0.43)	45.4 ± 4.94 (1)	6×

Values represent the averages of data from three assays. The standard deviation was calculated using a scientific calculator. For further information on the dNTP pool assay, see Materials and Methods.

*Ratio for each pool/total dNTP in each cell.

controls. In the M2-D clone, however, the total dNTP expansion was based primarily on dCTP and dGTP expansion. The dATP pool decreased to about half that of either dCTP or dGTP. In the X-D clone, dATP decreased further to only 8% of the total pool level and was about one-third that of either dCTP or dGTP. The dTTP pool remained at the same level as M2-D. The elevation in dCTP at the cost of a reduction in dTTP was unique to M2-D and X-D clones.

HUR cells showed the most deviation in dNTP values. The HUR clone selected from drug treatment possessed an increased RR activity and total dNTP pool that was nearly six-fold that of KB wild-type cells. The ratio of the dATP or dTTP individual pool to the total dNTP pool was similar to those in M2-D and X-D. Most notably, dCTP was expanded significantly to 30% of the total dNTP pool. As in the HUR cell line, the dATP pool was approximately one-half that of dCTP; however, dGTP was also about half that of dCTP, albeit within a large standard deviation. The dCTP pool in the HUR clone was greater than the corresponding pools in the M2-D and X-D clones. Figure 2a represents the concentration value of dNTP of each cell in Table 2 in a bar graph.

Figure 2b shows the relative ratio of dNTPs in each cell line compared with that of KB wild-type cells. The KB-V clone had the least-altered pool profile compared with the KB wild-type cells. M1-D clones demonstrated a remarkable six-fold increase in dATP levels and reciprocally decreased dTTP and dGTP levels. In the M1-D clone, the relatively lower degree of elevation of dCTP (4-fold) is noteworthy. The M2-D and X-D clones demonstrated an elevation in dCTP, with values 6- and 11-fold that of KB wild-type cells, respectively. Compared with dCTP, all the dATP, dGTP, and dTTP pools in M2-D and X-D clones were expanded 2- to 5-fold from those of the KB wild-type cells. The HUR clone demonstrated a marked expansion of dCTP to 15-fold that of KB wild-type cells. The dATP, dGTP, and dTTP pools in the HUR clone remained approximately 3- to 5-fold greater than the corresponding pools in KB wild-type cells. The marked increases in the dCTP pools of the M2-D, X-D, and HUR clones were proportional to their respective increases in RR activity.

Effect of HU on RR Activity in KB Wild-Type, KB-V, M1-D, M2-D, X-D, and HUR Clones

To understand whether the different properties of the RR enzyme alter the pool size, we examined the RR enzyme derived from all cell lines. The RR activity levels and sensitivities to HU were compared.

Various concentrations of HU were used as an inhibitor of RR in cell-free extracts from the various cell lines. The results are shown in Fig. 3. The cell-free extracts were treated with various concentrations of HU for 30 min prior to the assay of RR activity. Slight inhibition of RR activity was detectable in each cell line after samples were exposed to 0.001 and 0.01 mM of HU. From 0.01 to 0.1 mM, the average RR inhibition reduced enzymatic activity to 80 and 50% of the control, respectively, as shown in Fig. 3. The RR activity was depleted preferentially at a concentration of 0.1 to 1 mM. As the concentration of HU was increased, a further decrease in enzyme activity was observed. The enzyme activity decreased to about 40% at 0.1 mM and 60% at 1 mM. However, the patterns of inhibition were very similar among these cells. CDP reductase activity from the six cell lines seemed to be equally sensitive to inhibition by all concentrations of HU.

Effects of dNTP on RR Activity

The effects of different dNTP inhibitors on RR activity were studied using various cell lines. In the absence of dNTP inhibitors, RR activity using CDP as substrate was determined and set as 100%. The observed activities in the presence of inhibitor were expressed as a percentage of the control activity in each experiment, and these percentages were plotted against the concentration of inhibitors. The RR activity indicated by CDP reduction was measured at 30 min post-inhibition in the presence of 0.01, 0.1, 1, and 10 mM of dNTP, as shown in Fig. 4.

In KB wild-type cells, stimulation of RR activity by low levels of dCTP and dTTP was observed, but with increased dCTP and dTTP concentrations, inhibition of RR activity was seen instead. From quantitative comparison of the effect of each dNTP, RR activity showed no significant change when the concentration of dTTP was increased from 0 to 10 mM. The maximal inhibitory effect on the

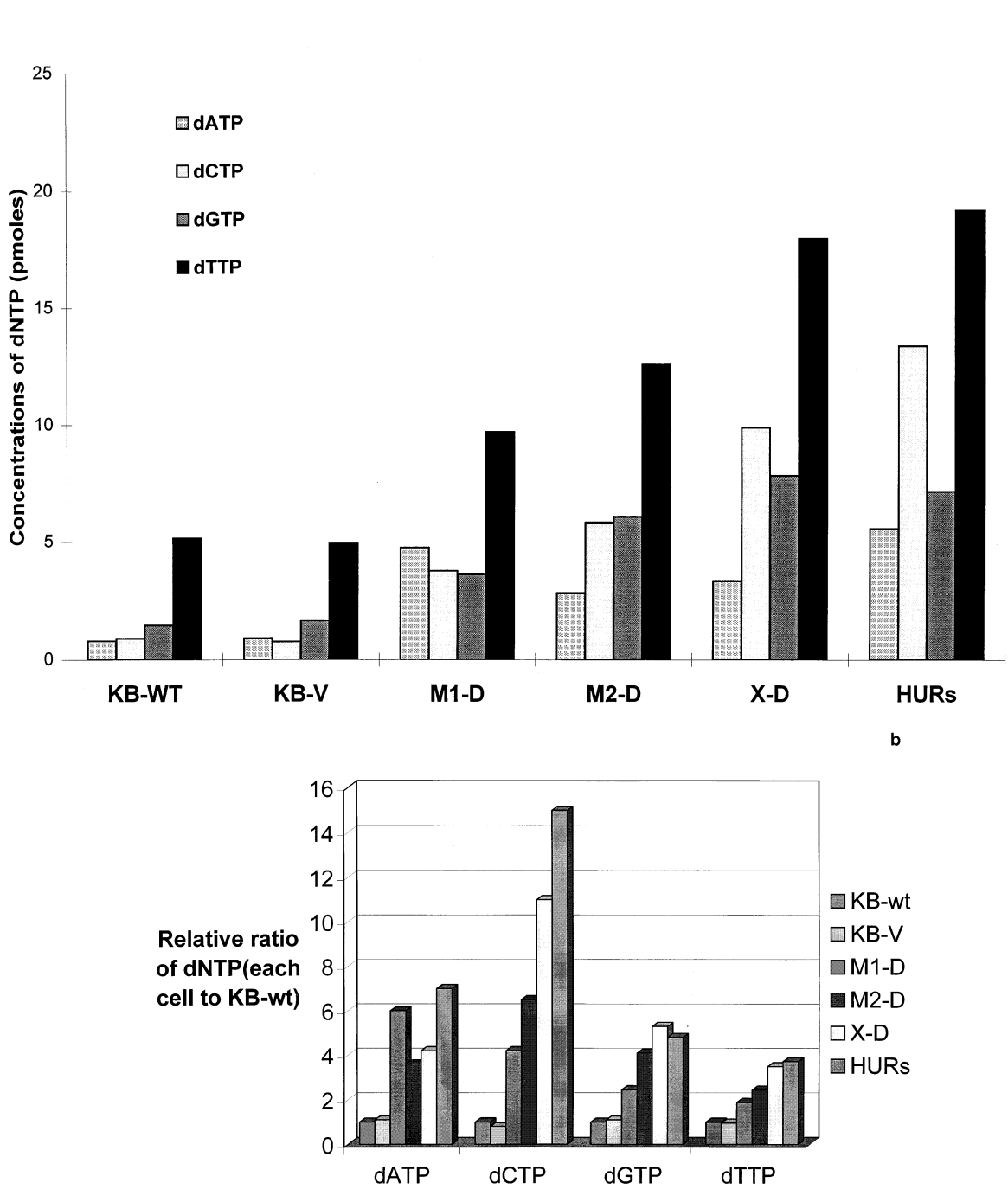

FIG. 2. (a) Concentration of dNTP pools in all cell lines relative to that in parental KB wild-type cells. Values for each cell line were analyzed from Table 2. (b) Relative ratio of dNTPs in each cell type to that of KB wild-type cells. The concentration value of dNTPs of each cell type used for this bar graph represents the data from Table 2. Columns in the key should be read from left to right in the figure, for each dNTP.

other dNTPs was observed at concentrations above 0.1 mM. It was observed that dCTP was the strongest inhibitor of RR activity and that it was effective at a much lower concentration than dATP and dGTP. At 1 mM, dATP and dGTP reduced RR activity to 60–80% of the control. However, at the same concentration, dCTP reduced RR activity to approximately 40%. At 10 mM, inhibition by dATP and dGTP steeply decreased RR activity to 30–40%, whereas dCTP inhibition resulted in 20% RR activity.

In the KB-V clone, dCTP inhibition of RR activity was again the most significant among the nucleoside triphosphates tested. It was inhibited by dCTP at a concentration of 0.1 mM. When the concentration was between 1 and 10 mM, dCTP inhibition reduced RR activity to 22%. In KB-V, dCTP and all other dNTP inhibition of RR activity revealed no significant change from that of KB wild-type cells.

M1-D clones behaved in a manner significantly different

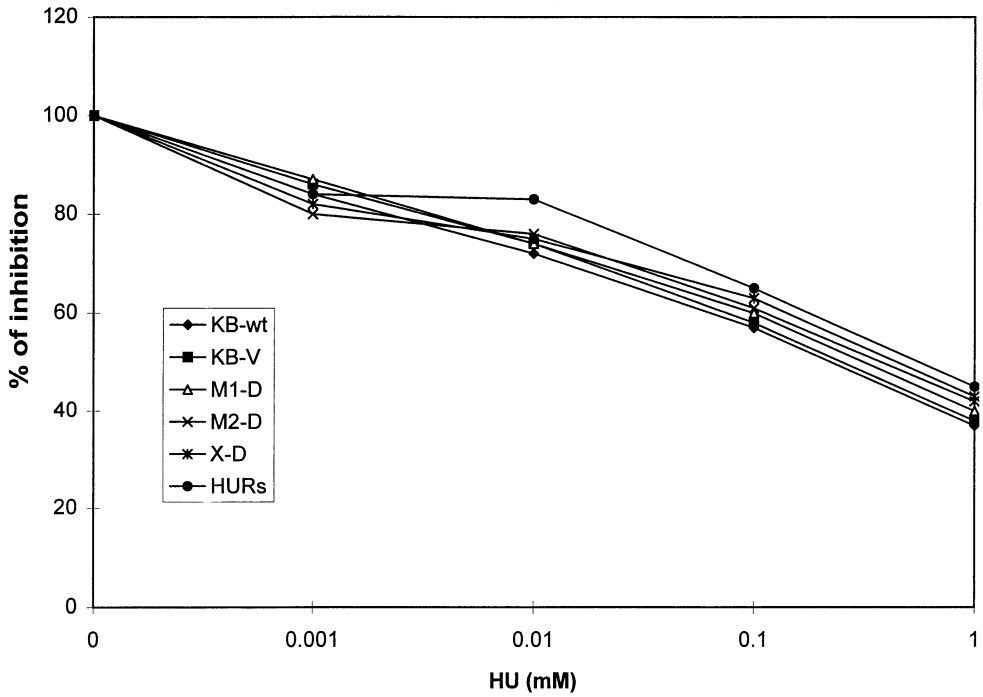

FIG. 3. Effects of HU on the enzyme activity for CDP reduction. Standard incubation conditions for CDP reduction were used in cell-free extracted protein from various cell types. Values are averages calculated from three separate experiments, each performed in duplicate.

from KB wild-type cells. First of all, concentrations at any level did not stimulate RR activity, as we observed in KB wild-type cells and KB-V clones. Second, there was no dNTP inhibition of RR activity that could be observed from concentrations of 0.01 to 1 mM. A slight inhibition was observed in the concentration range of 1 to 10 mM dNTP. dTTP inhibition could not be observed even at very high concentrations. The moderate inhibition from dGTP at concentrations up to 10 mM resulted in RR activity values of approximately 70%. This result apparently was very different from that in KB wild-type cells (30% from dGTP inhibition). Moreover, 10 mM of dATP could inhibit RR activity to only approximately 60%, which was much higher than its corresponding value of 40% in KB wild-type cells. At the same high concentration of dCTP, the inhibition was only 46% in M1-D clones versus 20% in KB wild-type cells. Overall, M1-D demonstrated a significant degree of resistance to dNTP inhibition of RR activity compared with control KB wild-type cells.

The dNTP inhibition patterns in M2-D, X-D, and HUR clones were very similar to each other but very different from M1-D and KB wild-type cells. When the concentration of dNTP was set at 0.1 mM, it was observed that dCTP gave rise to a 50% higher inhibition of RR activity than other dNTPs in M2-D, X-D, and HUR clones. In the presence of 1 mM of dCTP, RR activity was down to 20, 28, and 16% of controls in M2-D, X-D, and HUR cells, respectively. At 10 mM of dCTP, the activity level remained at 10% in M2-D and HUR clones; X-D remained at 16%. dATP levels at the 0.1-mM concentration, on the other hand, did not inhibit RR activity in these clones. At 1 mM of dATP, inhibition lowered RR activity to 70% in the M2-D and to 78% in the X-D clone but only to 85% in HUR cells. At higher concentrations of dATP, such as 10 mM, the inhibited enzymatic activity level ranged from 40 to 50% of the control among these clones.

Quantitatively, dATP inhibition of RR activity in M2-D, X-D, and HUR clones ranked directly between M1-D clones and KB wild-type cells in effectiveness. Compared with M2-D and X-D, the HUR clone exhibited a more profound inhibition by dCTP and a decreased sensitivity to dATP inhibition. The inhibition of RR activity with no obvious change in dTTP and dGTP pool sizes is also noteworthy. The M2-D and X-D clones were equally sensitive to dNTP inhibition of RR activity. However, dGTP inhibition was less effective in X-D than in M2-D or HUR cells. Overall, the X-D clone showed more resistance to dNTP inhibition than the M2-D clone, thus behaving as an M1-D clone.

DISCUSSION

Previous data from our laboratory suggest that HU resistance is due mostly to elevated amounts of M2 mRNA and increased RR activity. In this study, we have confirmed that an increase in RR activity is tightly correlated with total dNTP pool expansion as well.

The main finding of our experiment was that large dNTP pools are evidence of RR overexpression. Our results support the hypothesis that overproduction of M2 subunits

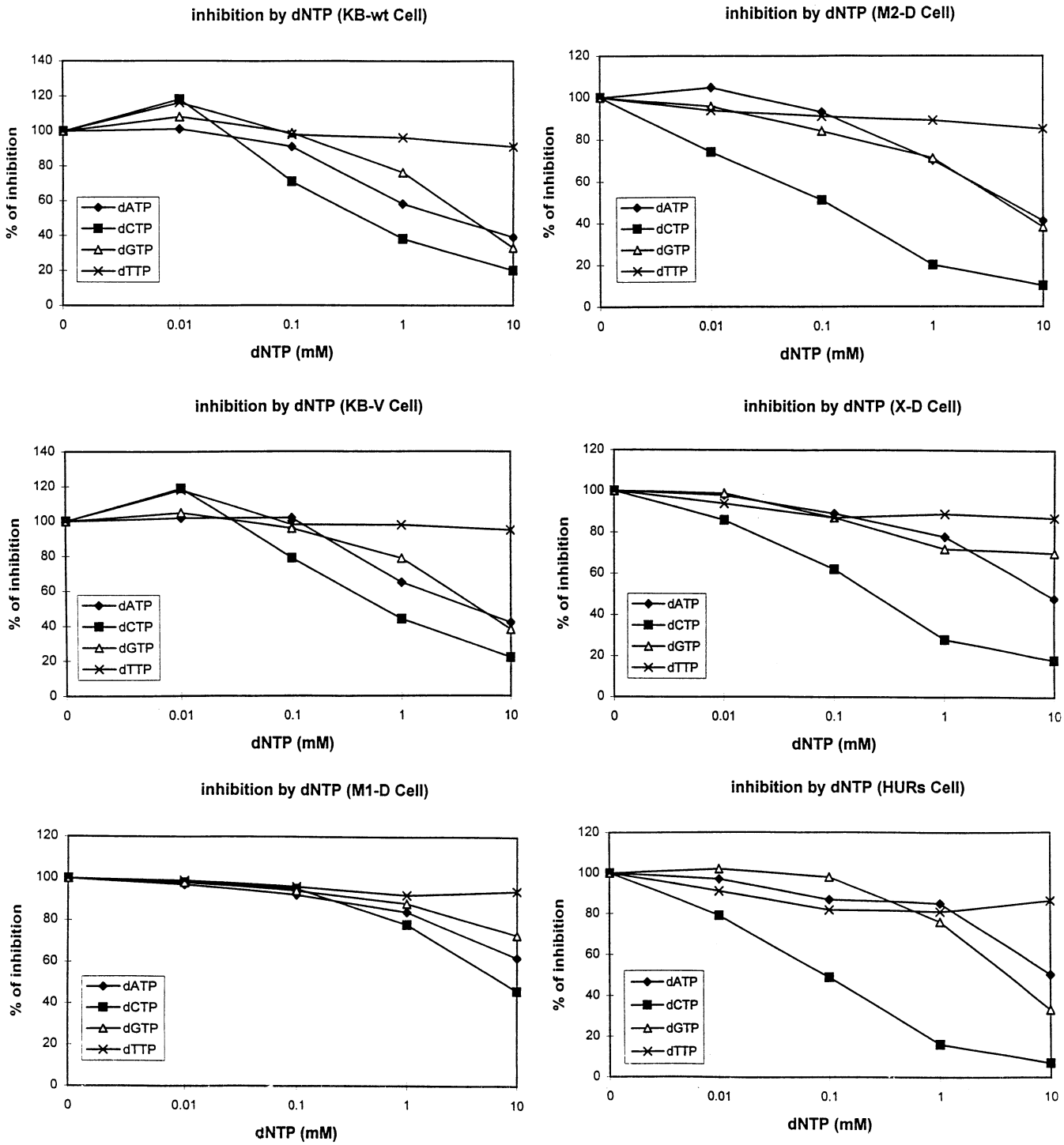

FIG. 4. Effects of different dNTPs on CDP reduction. The standard mixture containing the same amount of enzyme was incubated at various dATP concentrations with replacement by dTTP, dCTP, and dGTP. Each value represents the average of three separate experiments, each performed in duplicate.

confers HU resistance, which, in turn, increases dNTP pool sizes several fold by promoting reductase activity. In a preceding paper, we presented the three transfectant cells—M1-D, M2-D, and X-D—and reported that transfection of each distinct cDNA resulted in mRNA and protein overexpression for the appropriate subunit(s) only [14]. Overproduction of M1 mRNA and protein in M1 transfectant cells was not sufficient to increase HU resistance; M2 and X-D transfectant cells exhibited equal resistance to HU but neither matched the resistance levels of HUR cells. However, the upward progression of total dNTP levels (KB < M1-D < M2-D < X-D < HUR) and the dependence of dCTP production on the amount of active RR complexes present lead us to further postulate that HU resistance

correlates with dNTP, specifically dCTP, pool sizes as well as reductase activity.

It is accepted that in different cells, the distribution of each dNTP pool can be different ([1, 17] and Gao WY, Zhou BS, Johns DG, Mitsuya H and Ten Y, unpublished results). In mouse cell lines, the following results have been observed: slightly increased dTTP and dATP levels, a decreased dCTP pool, and no change in the dGTP pool [1]. Our studies on human cells demonstrate that the nearly identical pool sizes of dATP, dCTP, and dGTP in KB wild-type cells and KB-V clones contrast with the pool size variations apparent in M1-D, M2-D, X-D, and HUR cells. In our M2-D, X-D, and HUR clones, the most notable change is that the dCTP pool expanded to twice the size of the control, while the dATP pool shrank to one-half the size of dCTP. In contrast, in the M1-D clone, total dNTP level increases but elevation of dATP levels occurs at the cost of reductions in dCTP pools. Taking all the trends into consideration, dCTP pools enlarge most faithfully with increased RR activity.

Of special interest is the expansion of dATP pools due to M1 overexpression. It is possible that a decrease in dATP may reduce availability of positive effectors for UDP reduction and thus cause lower production of dGTP, which is activated by dTTP. dGTP merits closer scrutiny because it is the effector that promotes dATP synthesis [18–22]. Furthermore, the M1-D clone could owe its HU resistance to the accumulation of dATP (due to feedback inhibition of deoxynucleoside kinase by elevated dNTP pools) [23, 24]. Moreover, the accumulation of dATP could eventually turn off the synthesis of dTTP and dCTP. The depletion of dTTP could further affect the synthesis of dGTP. Thus, the intracellular concentration of dATP could be a critical factor in regulating RR activity [24, 25]. In other words, dATP could potentiate its own action by inhibiting other dNTP pools. The inhibition of dCTP and dGTP intracellular pools could, therefore, be a consequence, not a cause, of inhibited RR activity. Indeed, the suggestion has been made that M1-D clones resist dNTP inhibition of RR activity because the function of effector binding sites or the affinity of activator/inhibitor provisions is altered.

Enzyme kinetics are considered a consequence of potentiation by dNTPs on the binding affinity for RR activation/inhibition. Random cellular environmental factors could also lead any of the dNTP pools to show changes in RR activity. Indeed, dNTP pools emerge as an important factor in controlling RR activity and HU resistance of drug-induced HUR clones as well as transfectants. A more complete set of interactions between enzyme and dNTP pools probably exists. The integrity of RR function may require a precise balance between the concentrations of each pool intracellularly.

In summary, our findings support the following conclusions: (a) the observed expansion in dCTP and total dNTP pool sizes correlates closely with increased RR activity, and (b) increased levels of dCTP, total dNTP pool, and RR activity are signs of increased HU resistance.

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