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The Metabolic Basis of Deoxyuridine Cytotoxicity

Studies of Cultured Human Lymphoblasts

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SUMMARY

The metabolic consequences of deoxyuridine treatment in four cultured human lymphoblast lines (CCRF-CEM, RPMI-8402, JM, and BALM) were studied by cell growth experiments, flow cytometry, and measurement of 2′-deoxyribonucleoside triphosphate (dNTP) levels. DNA perturbations occurred in all lymphoblast lines, but there was no significant impairment of RNA synthesis. The DNA perturbations in CCRF-CEM, RPMI-8402, and BALM cells reflected inhibition of DNA synthesis, and the associated dNTP changes were consistent with ribonucleotide reductase inhibition or, specifically in BALM cells, with DNA α-polymerase inhibition. JM cells treated with an intermediate concentration of deoxyuridine developed a block at the G_1/S boundary which was deoxyuridine concentration-dependent, but not specific for deoxyuridine (it was also seen with thymidine treatment) and not related to DNA synthesis inhibition. There were no idiosyncratic dNTP effects accompanying the G_1/S boundary block, and the responsible metabolic mechanism remains to be determined.

INTRODUCTION

Since the discovery that the lymphocytotoxicity associated with some forms of primary specific immunodeficiency disease was mediated by elevated levels of purine nucleosides and nucleotides (1, 2), there has been general interest in the metabolic mechanisms of nucleoside cytotoxicity. Fox *et al.* (3) reported cytotoxicity studies in 10 cultured lymphoblast lines (leukemic and Epstein-Barr virus-transformed) treated with dThd, dCyd, dAdo, and dGuo. B lymphoblasts were approximately 100-fold less sensitive to dThd, dAdo, and dGuo than were T and null lymphoblasts, and all lymphoblasts, regardless of subtype, were relatively resistant to dCyd treatment.

The demonstration that ribonucleotide reductase activity was under the allosteric control of dNTPs[1] (4, 5) has provided the main metabolic framework for the understanding of dThd (6–8) and dGuo (9) cytotoxicities. The relationship between ribonucleotide reductase inhibition and dAdo toxicity is less well defined, and the only significant dNTP depletion seen with 3 μM dAdo was a delayed (8 hr after dAdo addition) fall in dGTP levels (10). The cytotoxic significance of this dGTP decrease was inconclusive, as was its relationship to allosteric inhibition of ribonucleotide reductase. Another explanation for dAdo toxicity is the inactivation of *S*-adenosylhomocysteine hydrolase with resultant inhibition of methylation reactions (11). An alternative comprehensive explanation for deoxyribonucleoside cytotoxicity (12, 13) postulates the interaction of elevated dNTP levels plus an intermediary (regulator) protein with DNA α-polymerase thereby causing this enzyme's inhibition.

Compared with the numerous biochemical studies of dThd, dAdo, and dGuo toxicities there is little information concerning the mechanisms of dUrd toxicity. This may reflect the fact that dUrd is usually thought of solely as a dTMP precursor and therefore dUrd toxicity is assumed to operate through dTTP. Another possible contributor to dUrd toxicity is incorporation of dUMP into DNA. Such incorporation has been demonstrated in mutant bacteria deficient in dUTPase or uracil-DNA glycosidase (14) and in association with methotrexate treatment (15). It is feasible that high dUTP levels associated with pharmacological concentrations of dUrd might result in significant dUMP incorporation into DNA.

We have investigated the metabolic consequences of dUrd treatment in four human cultured lymphoblast lines by measuring cell growth inhibition and dNTP levels and by analyzing cell DNA and RNA contents with flow cytometry.

MATERIALS AND METHODS

The human lymphoblast cell lines studied were CCRF-CEM, RPMI-8402, JM (all T lymphoblasts), and BALM (B lymphoblast), which were obtained from Dr. H. Lazarus, Sidney Farber Cancer Center (Boston, Mass.) and Dr. H. Zola, Flinders Medical Centre (Adelaide, Aus-

[1] The abbreviation used is: dNTP, 2′-deoxyribonucleoside triphosphate.

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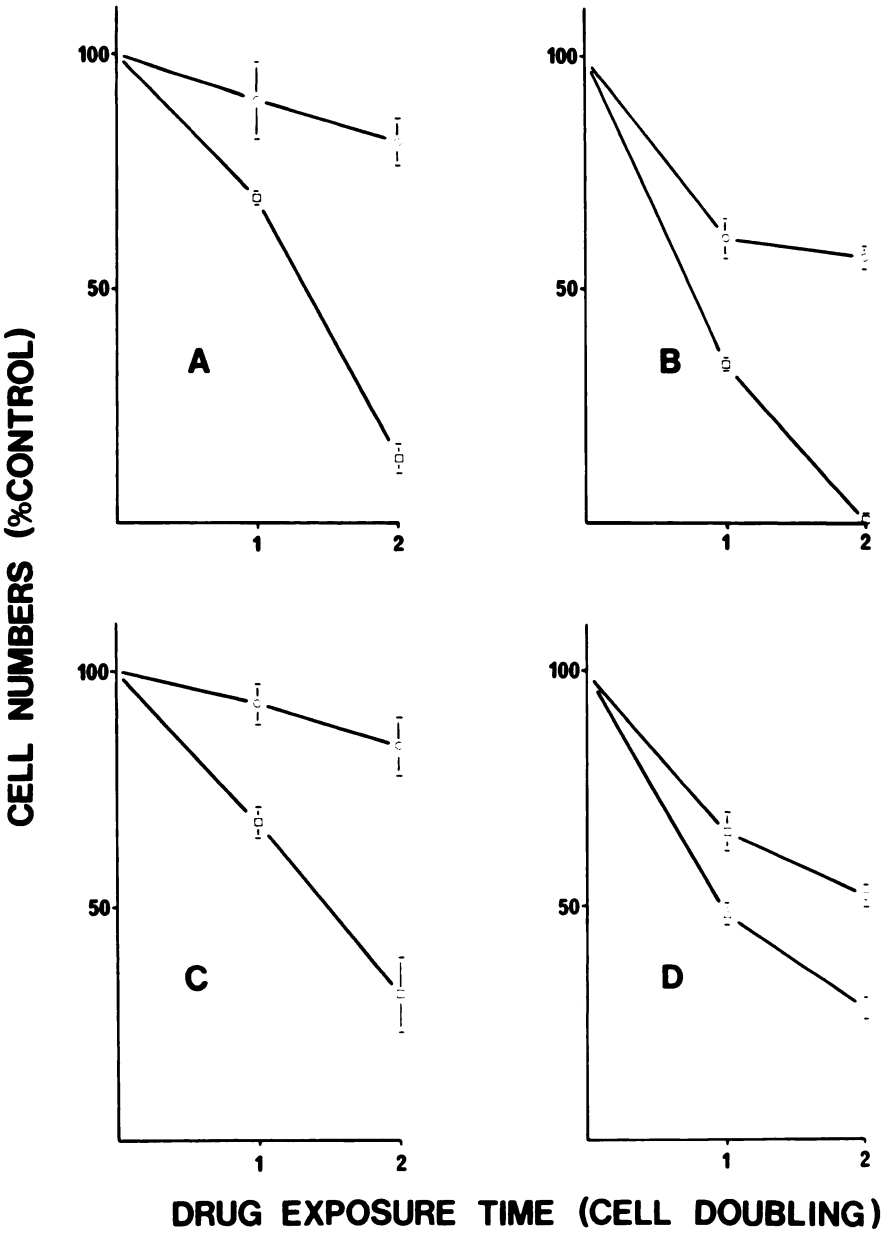

FIG. 1. *Lymphoblast growth with dUrd treatment*

A, CCRF-CEM; B, RPMI-8402; C, JM; D, BALM; ○, dUrd 10^{-4} M; □, 5×10^{-4} M; △, 10^{-2} M. Cells were established at a cell concentration of 2×10^5/ml, and drugs were added when cell number was 2×10^5/ml. Results are the means of at least three experiments.

tralia). Cells were grown in suspension culture in RPMI 1640 plus 10% fetal calf serum (Flow Laboratories, New Haven, Conn.), and in logarithmic phase growth the cell-doubling times were 24 hr (CCRF-CEM and JM) and 36 hr (RPMI-8402 and BALM). Twenty-four hours prior to the addition of nucleosides (all of which were obtained from the Sigma Chemical Company, St Louis, Mo.), the cells were suspended in fresh medium at a concentration of 10^5/ml. All cells counts were performed manually on a Neubauer hemocytometer. Metabolic studies were performed at times related to the cell doubling time, viz: 0.05, 0.25, 0.5, 1, and 2 cell-doubling times after the addition of nucleoside.

dNTPs were measured by the DNA polymerase method of Solter and Handschumacher (16) with modifications by Lindberg and Skoog (17). Nucleotides were extracted by incubating 5×10^6 washed cells with 60% ethanol at 30° for 10 min. Results are the means of duplicate assays from two separate experiments.

For single-parameter DNA content analysis, cells were stained with ethidium bromide and mithramycin as described by Taylor and Milthorpe (18). For dual-parameter DNA/RNA content analysis, cells were stained with the two-step acridine orange procedure described by Traganos *et al.* (19). All analyses were performed on an ICP22 flow cytometer (Ortho Instruments, Westwood, Mass.).

RESULTS

The growth-inhibitory effects of dUrd in the four lymphoblast lines are shown in Fig. 1. Higher dUrd concentrations were required in the BALM (B lymphoblast)

line to achieve growth inhibition comparable to that seen with T lymphoblasts. RPMI-8402 cells were most sensitive to dUrd, and complete cell kill was seen after treatment for 2 cell-doubling times.

To determine the effects of dUrd on DNA and RNA metabolism, single-parameter DNA and dual-parameter DNA/RNA analyses were performed. The single-parameter DNA histograms from CCRF-CEM cells are shown in Fig. 2. dUrd 5×10^{-4} M produced a modest decrease in S-phase cells, a more pronounced loss in G_2+M, and an increase in G_1 cells. Less marked perturbations occurred with dUrd 10^{-4} M treatment. A similar pattern was seen with RPMI-8402 cells (Table 1), and at 1 cell-doubling time the histogram of the cells treated with dUrd 10^{-4} M was reverting to normal with a cohort of cells passing through the S and G_2+M phases. The DNA changes in these two cell lines are consistent with partial or complete impairment of DNA synthesis which effectively halted progression of S-phase cells through the cell cycle. The decrease of G_2+M cells and increase of G_1 cells indicates progression of cells between these compartments. The BALM results also shown in Table 1 reveal an initial accumulation of cells in the S-phase dUrd 10^{-2} M followed by release of the arrested cells (1 cell-doubling time) and progression of a cell cohort through late S and G_2+M phases. This pattern is typical of incomplete inhibition of DNA synthesis.

A different type of DNA perturbation was seen with JM cells (Table 1). The effects of dUrd 10^{-4} M were minimal, in keeping with the minor growth inhibition

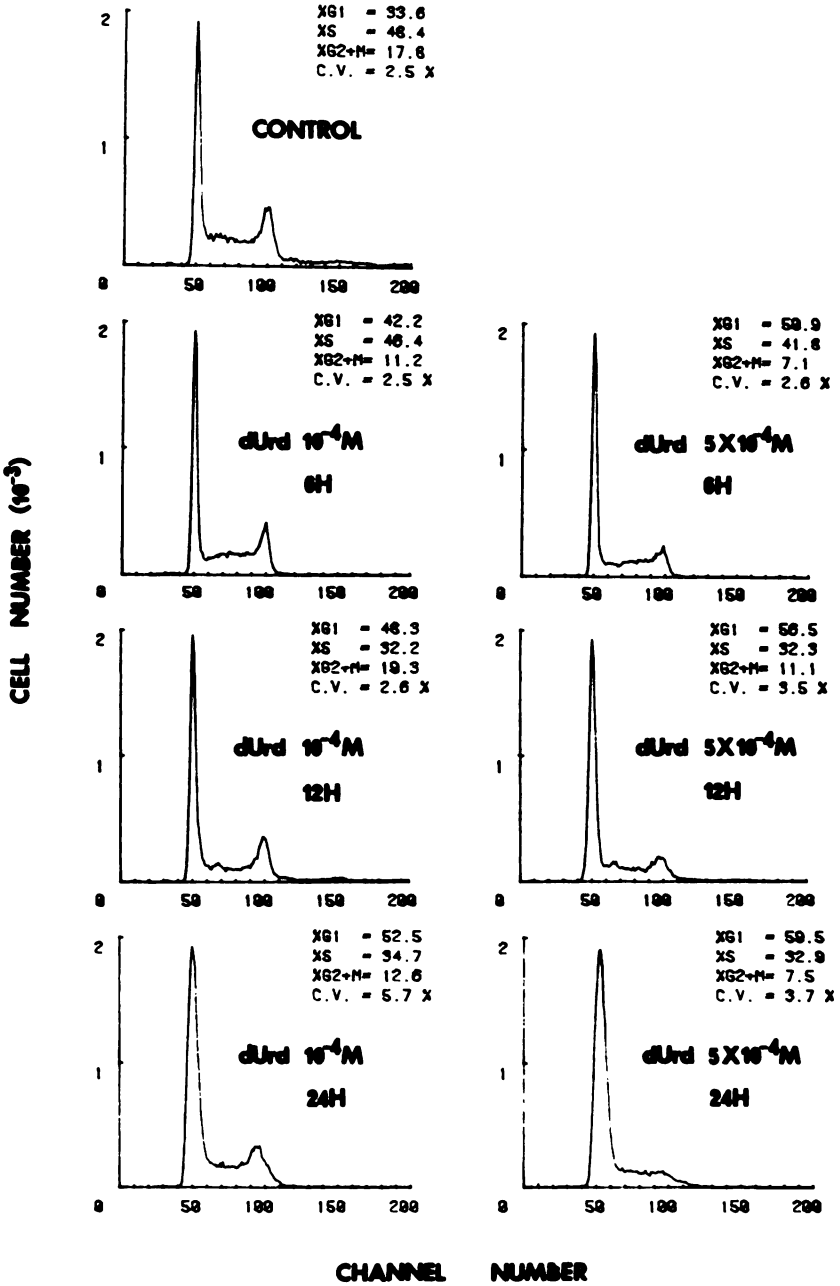

FIG. 2. *DNA content analysis of CCRF-CEM cells treated with dUrd* G_1 peak was set at channel 50. *C.V.*, coefficient of variation.

TABLE 1

Comparison of DNA content analyses of the RPMI-8402, BALM, and JM cells treated with varying dURd concentrations for varying periods

Cells	Control %			dURd concentration	Time								
					9 Hr			18 Hr			36 Hr		
	G_2	S	G_2 + M		G_1	S	G_2 + M	G_1	S	G_2 + M	G_1	S	G_2 + M
				M									
RPMI 8402	27	56	16	10^{-4}	45	46	9	47	44	9	27	52	21
RPMI 8402				5×10^{-4}	48	47	5	48	51	1	50	46	4
BALM	34	44	22	5×10^{-4}	32	44	24	28	49	23	29	48	23
BALM				10^{-2}	26	69	15	11	69	20	21	54	25
					6 Hr			12 Hr			24 Hr		
					G_1	S	G_2 + M	G_1	S	G_2 + M	G_1	S	G_2 + M
JM	39	45	15	10^{-4}	42	45	13	38	46	16	35	44	20
JM				5×10^{-4}	64	19	17	71	19	10	0	0	7

observed. dUrd 5×10^{-4} M treatment produced a loss of early S-phase cells which progressed to a severe loss of S and G_2+M cells. The resulting accumulation of cells in G_1 appeared to be resolving by 1 cell-doubling time as cells moved in cohort into the S-phase. This pattern is consistent with a block to cell progression near the G_1/S boundary associated with unimpeded progression through S and G_2+M. An alternative explanation for this phenomenon might be the selective killing of S and G_2+M cells with accumulation of cells in G_1 because of completely suppressed DNA synthesis. This latter hypothesis should be associated with reduced cell numbers; however, a small and consistent increase in cell numbers was observed, thus favoring the G_1/S boundary block theory. To determine whether the G_1/S boundary effect was dUrd concentration-dependent or dUrd-specific, JM cells were treated with dUrd 1.5×10^{-3} M and 10^{-2} M and dThd 10^{-2} M. The G_1/S boundary effect was seen with dUrd 1.5×10^{-3} M (results not shown), but with 10^{-2} M (Fig. 3) a "frozen S-phase" effect was seen similar to the results with CCRF-CEM and RPMI-8402 cells. dThd 10^{-2} M treatment (a concentration equitoxic with dUrd 5×10^{-4} M) caused the characteristic G_1/S boundary effect (Fig. 3).

The presentation of the correlated DNA/RNA analyses (Figs. 4 and 5) is limited to 1 cell-doubling time studies because the abnormalities were most evident at this time. The steplike lines drawn on each contour represent the limits of the unperturbed cell contour. The contour plots of CCRF-CEM, RPMI-8402, and JM cells treated with dUrd 5×10^{-4} M show marked depletion of cells with a greater than G_1 DNA (green fluorescence) content and an abnormally high RNA (red fluorescence) content in the residual G_1 cells. This pattern reflects continuing RNA synthesis in cells whose passage through the DNA synthetic phase was delayed or blocked. dUrd 10^{-4} M treatment produced changes of lesser severity or, in the case of JM cells, no perturbation at all. The BALM contour plots (Fig. 5) show preservation of cell cycle phases with a moderate increase in G_2+M cell RNA content.

These flow cytometric data show cell cycle perturbations in all four lymphoblast lines treated with dUrd without associated inhibition of RNA synthesis. The changes seen in CCRF-CEM, RPMI-8402, and BALM cells suggest inhibition of DNA synthesis; however, the G_1/S boundary block seen in JM cells with lower dUrd concentrations appeared to be independent of DNA synthesis inhibition.

The dNTP pool changes in CCRF-CEM cells treated with dUrd are shown in Fig. 6. Note that the designation dTTP includes both dTTP and dUTP, since the DNA polymerase assay, with poly(dA-dT) as template, cannot discriminate dTTP from dUTP. Dramatic, linear increases in dTTP, dGTP, and dATP were seen with dUrd 5×10^{-4} M, and a lesser response occurred with dUrd 10^{-4} M. However, an inverse pattern was seen with dCTP levels, and there was slight recovery from the nadir (1 cell-doubling time) produced by dUrd 5×10^{-4} M. Generally, similar dNTP changes occurred in RPMI-8402 cells (Fig. 7) and again there was a late recovery from initially depressed dCTP levels.

Because the low dUrd concentrations produced minimal derangement of JM and BALM dNTP levels, the dNTP assays were performed more frequently to detect transient dNTP changes in the initial phases of treatment. The dTTP increase in JM cells (Fig. 8) with dUrd 5×10^{-4} M was less marked than that seen with CCRF-CEM and RPMI-8402 cells. This is consistent with the fact that JM cells are thymidine kinase mutants. A moderate and transient depression of dCTP levels was produced by dUrd 5×10^{-4} M, but no decrease occurred with the lower concentration. dATP and dGTP increases were similar to those seen in the other T lymphoblast lines. Bearing in mind the higher dUrd concentrations used in the B cell line, BALM, the dTTP and dGTP changes (Fig. 9) were similar in magnitude to those seen in the T lymphoblasts. The significantly different effects were the blunted increase in dATP levels, the small and transient depression of dCTP levels, the small and transient depression of dCTP levels with dUrd 10^{-2} M despite a dramatic dTTP rise, and the lack of dCTP depression with low-concentration dUrd. Conversely, dCTP levels rose linearly with dUrd 5×10^{-4} M to 315% of control by the 2 cell-doubling time point.

To assess the relationship between depressed dCTP levels and growth inhibition, cells were simultaneously treated with dUrd and dCyd. Fox *et al.* (8) reported

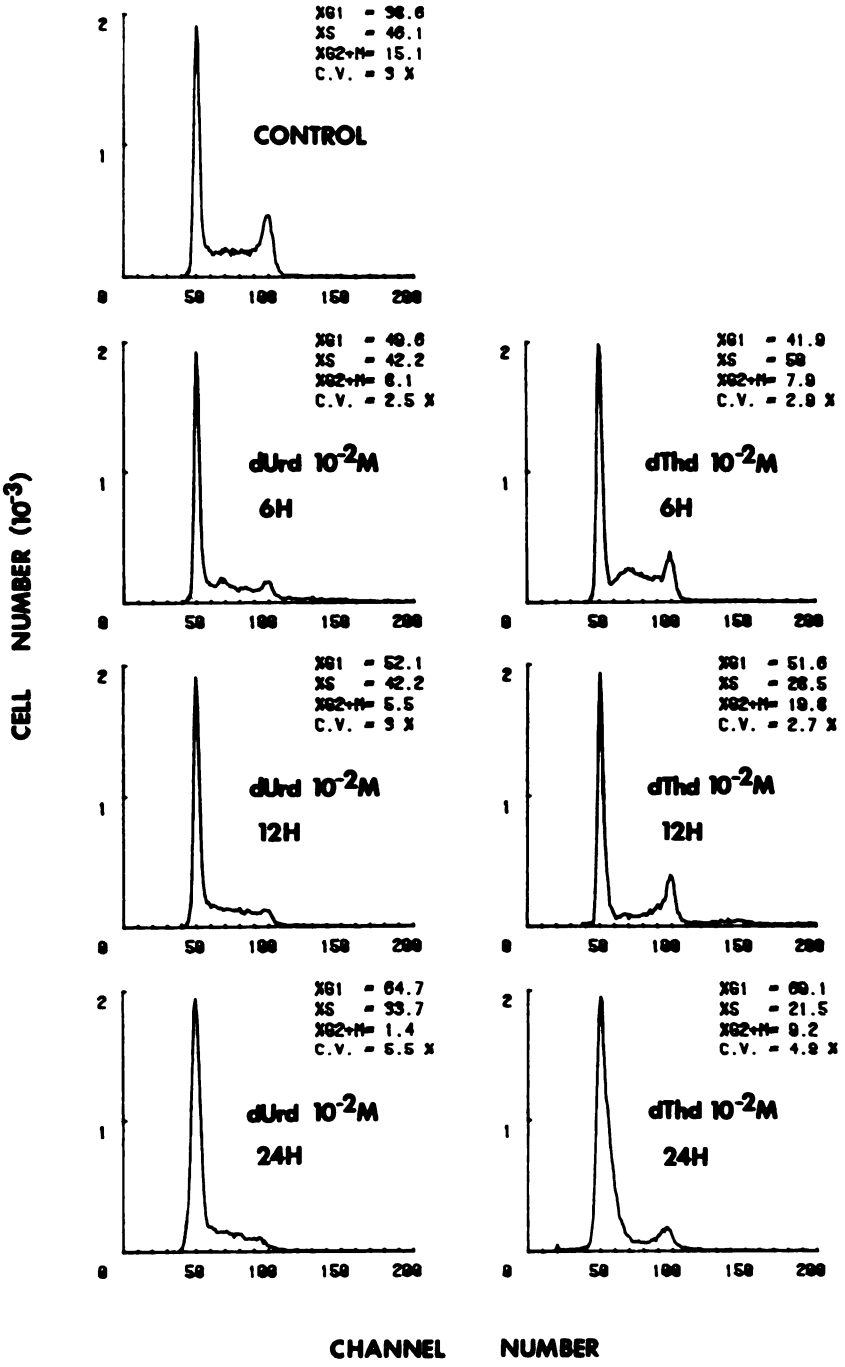

FIG. 3. *DNA content analysis of JM cells treated with dUrd and dThd* G_1 peak was set at channel 50. *C.V.*, coefficient of variation.

complete prevention of TdR toxicity in CCRF-CEM cells by the addition of dCyd 10^{-5} M. Preliminary experiments showed that the B lymphoblast line, BALM, was not rescued with this concentration, so dCyd 10^{-3} M was used with this cell line. The results of the rescue experiments are shown in Fig. 10. The dUrd-induced inhibition of CCRF-CEM, RPMI-8402, and JM cell growth was virtually abolished by the addition of dCyd. The reason for the slight JM growth inhibition produced by dCyd alone at 2 cell-doubling times is unclear, but this effect probably limited complete dCyd reversal of dUrd toxicity at this time point. dCyd 10^{-3} M produced incomplete sparing of dUrd growth inhibition in BALM cells, and the rescue was greater with dUrd 10^{-2} M than with 5×10^{-4} M. dCyd 10^{-3} M was itself slightly toxic, as was a lower concentration (10^{-4} M). dNTP pool measurement in CCRF-CEM and JM cells exposed to dUrd (5×10^{-4} M) and dCyd (10^{-5} M) indicated that dCyd corrected the dCTP pool depression caused by dUrd in both cell lines (data not shown). dCyd reduced the dTTP pool elevation caused

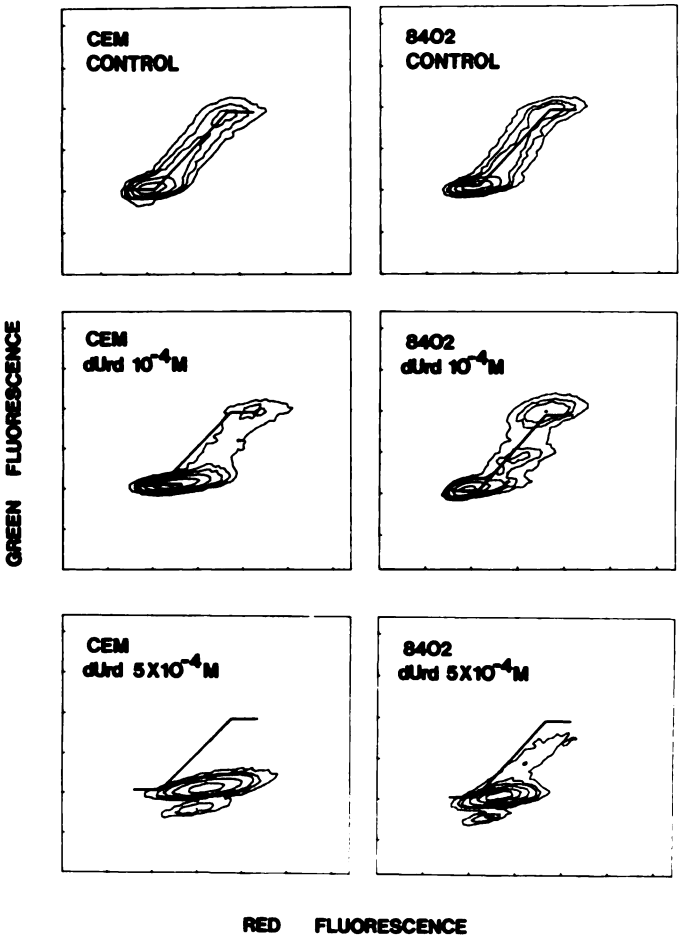

FIG. 4. *Correlated DNA-RNA contour plots of cells treated with dUrd*

The steplike line represents the limits of the untreated cells' contour. *Green fluorescence* and *red fluorescence* represent DNA and RNA contents, respectively.

by dUrd to a greater extent in the CCRF-CEM than in the JM (883% → 317% in CCRF-CEM versus 461% → 219% in the JM).

DISCUSSION

The results reported here indicate that the metabolic mechanisms of dUrd toxicity in cultured human lymphoblasts are diverse. This observation parallels other reports of heterogeneous metabolic responses associated with dThd (9, 20) and dAdo (10) treatments. The metabolic consequences of dUrd treatment in these lymphoblast lines were variably lymphoblast line-specific and dUrd concentration-dependent.

The dNTP changes seen in CCRF-CEM, RPMI-8402, and JM cells treated with dUrd were consistent with allosteric regulation of ribonucleotide reductase by dNTPs (4, 5). Elevated dTTP levels allosterically activate GDP reduction and inhibit CDP reduction. The resulting elevation of dGTP levels allosterically activates ADP reduction. The reversal of the initial dCTP depression seen in all of the T lymphoblast lines is difficult to explain in view of the persistent dTTP elevations. Possible explanations include salvage of dCyd from dead cells and metabolic de-inhibition of ribonucleotide reductase due to interaction of the changing relative concentrations of dNTPs (21).

The growth of BALM cells treated with a low concentration (5×10^{-4} M) of dUrd was 50% inhibited at 2 cell-doubling times yet there was no associated dNTP depletion. On the contrary, dTTP, dCTP, dATP, and dGTP levels rose to 460%, 315%, 326%, and 223% of control, respectively, after 2 cell-cycle times treatment. Since Reichard (22) has shown that dCTP levels in mouse embryo cells were linearly related to the proportion of S-phase cells, it is possible that a genuine dCTP depletion might be concealed by a drug-induced accumulation of S-phase cells. This argument is unlikely to apply to BALM cells treated with dUrd 5×10^{-4} M because the S-phase increased by only 5%. In the absence of dNTP depletion and (by implication) ribonucleotide reductase inhibition, alternative explanations for low-concentration dUrd toxicity in this cell line include inhibition of DNA α-polymerase by elevated dNTP levels (12, 13) and increased incorporation of dUMP into DNA in association with elevated dUTP levels (15).

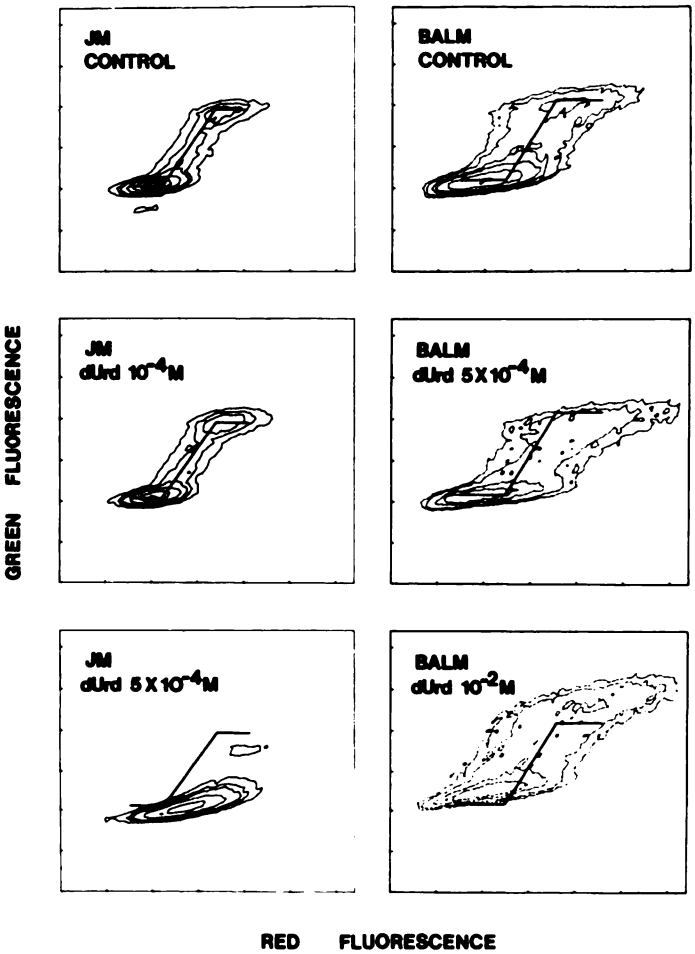

FIG. 5. *Correlated DNA-RNA contour plots of cells treated with dUrd*

The steplike line represents the limits of the untreated cells' contour. *Green fluorescence* and *red fluorescence* represent DNA and RNA contents, respectively.

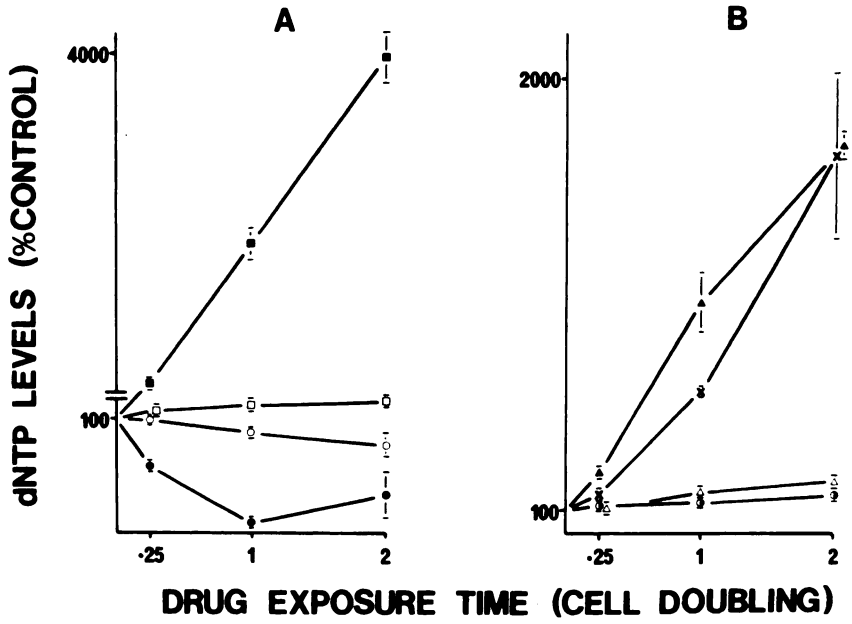

FIG. 6. *dNTP levels in CCRF-CEM cells treated with dUrd*
A. □, dTTP (dUrd 10^{-4} M); ■, dTTP (dUrd 5×10^{-4} M); ○, dCTP (dUrd 10^{-4} M); ●, dCTP (dUrd 5×10^{-4} M).
B. △, dATP (dUrd 10^{-4} M); ▲, dATP (dUrd 5×10^{-4} M); ◑, dGTP (dUrd 10^{-4} M); ×, dGTP (dUrd 5×10^{-4} M).
Control dNTP levels (picomoles/10^6 cells) at 24 and 48 hr, respectively, were: dATP, 7.1 and 8.5; dTTP, 5.3 and 6.4; dCTP, 4.4 and 5.1; dGTP, 6.3 and 5.7.

The dNTP changes seen in BALM cells treated with dUrd 10^{-2} M were similar to those seen in the T lymphoblast lines except that the dCTP decrease was modest and transient and the dATP increase was blunted. The modest dCTP depression may have been related to the unimpressive dATP increase and it could be argued that the dCTP depletion was insufficient to account for the observed growth inhibition (20). In this case the alternative mechanisms mentioned above could be invoked to explain the difference. The progressive elevation of dTTP levels over 2 cell cycles differs from the observation of Carson *et al.* (23) that dTTP levels following dThd treatment in the B lymphoblast, Wil-2, reached their peak at 6 hr and then declined.

The flow cytometric data demonstrated cell cycle perturbations in all lymphoblast lines exposed to dUrd (excepting JM cells treated with a low concentration of dUrd), but there was no evidence of significant RNA synthesis inhibition. The cell cycle perturbations seen with CCRF-CEM, RPMI-8402, and BALM cells were consistent with time- or concentration-dependent inhibition of DNA synthesis. However, the apparent G_1/S

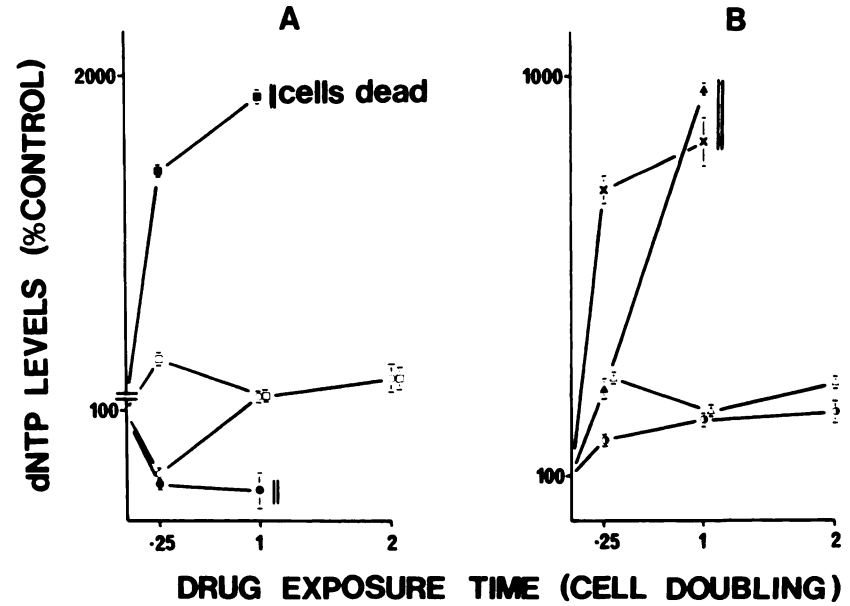

FIG. 7. *dNTP levels in RPMI-8402 cells treated with dUrd*
A. □, dTTP (dUrd 10^{-4} M); ■, dTTP (dUrd 5×10^{-4} M); ○, dCTP (dUrd 10^{-4} M); ●, dCTP (dUrd 5×10^{-4} M).
B. △, dATP (dUrd 10^{-4} M); ▲, dATP (dUrd 5×10^{-4} M); ◑, dGTP (dUrd 10^{-4} M); ×, dGTP (dUrd 5×10^{-4} M).

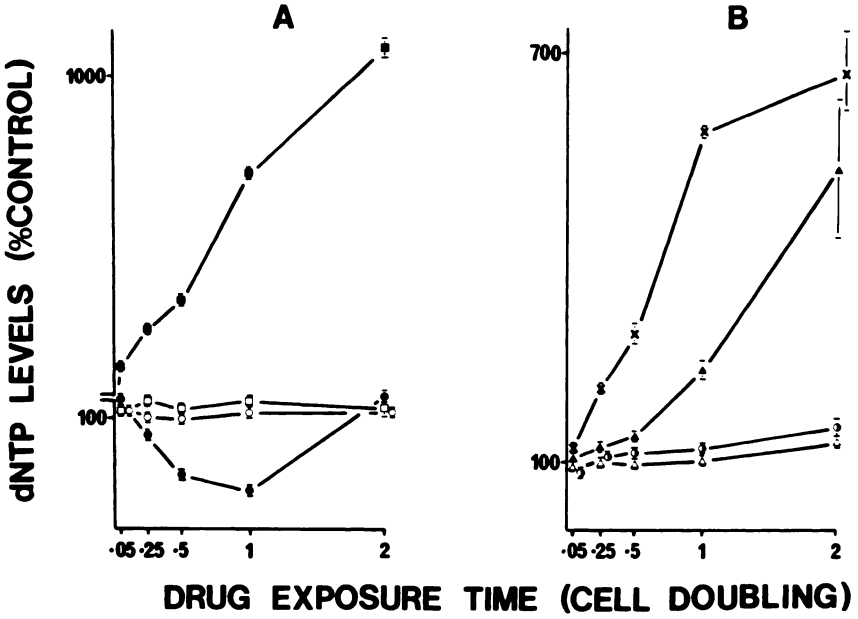

FIG. 8. *dNTP levels in JM cells treated with dUrd*
A. □, dTTP (dUrd 10^{-4} M); ■, dTTP (dUrd 5×10^{-4} M); ○, dCTP (dUrd 10^{-4} M); ●, dCTP (dUrd 5×10^{-4} M).
B. △, dATP (dUrd 10^{-4} M); ▲, dATP (dUrd 5×10^{-4} M); ◑, dGTP (dUrd 10^{-4} M); ×, dGTP (dUrd 5×10^{-4} M).
Control dNTP levels (picomoles/10^6 cells) at 24 and 48 hr, respectively were: dATP, 12.5 and 18.1; dTTP, 8.2 and 11.7; dCTP, 5.6 and 7.5; dGTP, 8.7 and 9.0.

boundary block pattern seen in JM cells treated with dUrd 5×10^{-4} M to 1.5×10^{-3} M could not be related to DNA synthesis inhibition because the progression of S-phase cells was not impeded. This phenomenon did not reflect selective killing of S-phase cells because cell numbers initially increased following the addition of dUrd. A similar dose-dependent G_1/S boundary effect has been reported in CCRF-CEM cells treated with dAdo 3×10^{-6} M in the presence of an inhibitor of adenosine deaminase (10).

The dNTP data from JM cells treated with dUrd 5×10^{-4} M were consistent with ribonucleotide reductase inhibition and did not contain any idiosyncratic explanation for the flow cytometric effect. The dNTP data related to the dAdo-induced G_1/S block in CCRF-CEM cells (10) showed a delayed fall in dGTP levels, a minor fall in dTTP, and elevation of dATP and dCTP. Thus the only dNTP factor common to CCRF-CEM/dAdo and JM/dUrd data was the elevation of dATP. The dAdo-induced CCRF-CEM growth inhibition and G_1/S boundary block were prevented by addition of dCyd 10^{-4} M, and, although dCyd 10^{-5} M also reversed the growth

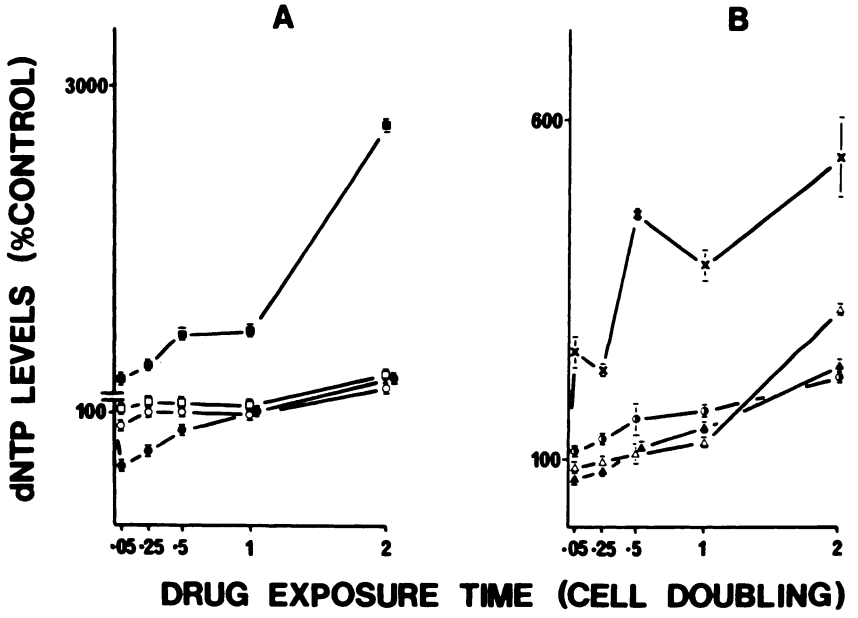

FIG. 9. *dNTP levels in BALM cells treated with dUrd*
A. □, dTTP (dUrd 10^{-4} M); ■, dTTP (dUrd 5×10^{-4} M); ○, dCTP (dUrd 10^{-4} M); ●, dCTP (dUrd 5×10^{-4} M).
B. △, dATP (dUrd 10^{-4} M); ▲, dATP (dUrd 5×10^{-4} M); ◑, dGTP (dUrd 10^{-4} M); ×, dGTP (dUrd 5×10^{-4} M).

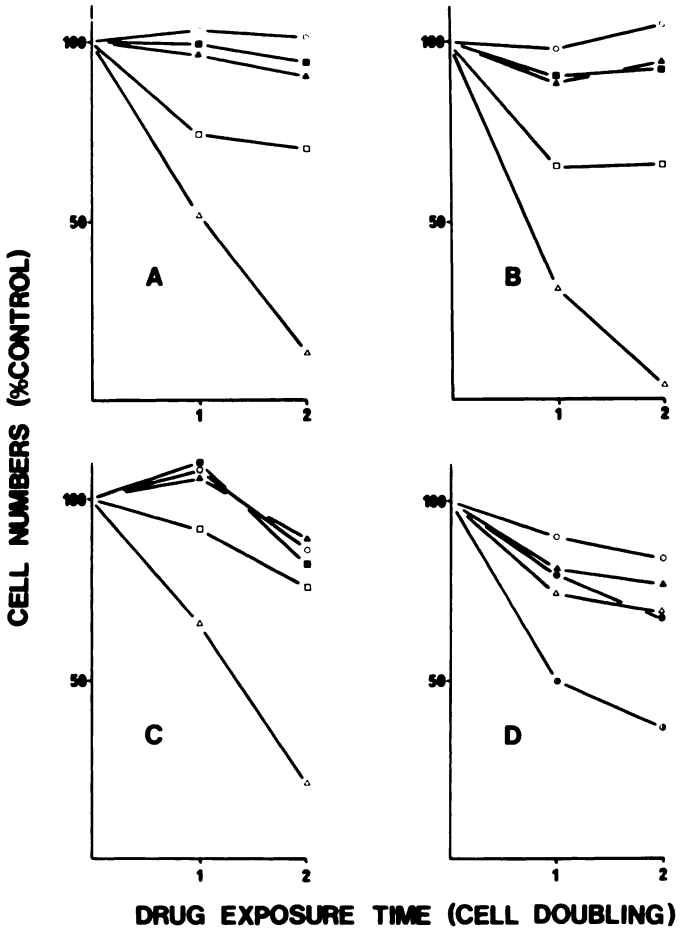

FIG. 10. *Lymphoblast growth with simultaneous dUrd and dCyd treatment*

A, CCRF-CEM; B, RPMI-8402; C, JM; D, BALM; ○, dCyd 10^{-5} M (CCRF-CEM, RPMI-8402, and JM) and dCyd 10^{-3} M (BALM); □, dUrd 10^{-4} M; △, dUrd 5×10^{-4} M; ◑, dUrd 10^{-2} M; ■, dUrd 10^{-4} M plus dCyd 10^{-5} M; ▲, dUrd 5×10^{-4} M plus dCyd 10^{-5} M; ●, dUrd 10^{-2} M plus dCyd 10^{-3} M. Live cells were identified under phase-contrast microscopy by their refractile properties and morphology.

inhibition of JM cells produced by dUrd 5×10^{-4} M, it cannot be inferred that the G_1/S block was necessarily dCTP- or dNTP-related.

The metabolic mechanism for the G_1/S boundary block remains to be determined. Because DNA synthesis inhibition was not an associated feature, the effect is unlikely to be related to ribonucleotide reductase or DNA α-polymerase inhibition. Since elevated dATP levels were common to JM and CCRF-CEM cells exhibiting the G_1/S boundary block, it is conceivable that dATP could allosterically inhibit an enzyme involved in the G_1 to S-phase transition. The concentration dependence of the block could then be explained by metabolic de-inhibition resulting from changing relative concentrations of nucleotides. If these assumptions are correct, then the G_1/S block might occur in other cell lines treated with an appropriate concentration of dUrd, dThd, or dAdo or with other nucleosides which produce elevated dATP levels.

In summary, the metabolic mechanisms of dUrd cytotoxicity in CCRF-CEM, RMPI-8402, JM, and BALM lymphoblast lines include DNA synthesis inhibition and a G_1/S boundary block which is not related to DNA synthesis inhibition. The mechanisms of DNA synthesis inhibition include ribonucleotide reductase-mediated dNTP depletion and possibly inhibition of DNA α-polymerase. The G_1/S boundary effect is neither lymphoblast line-specific nor nucleoside-specific and may be related to elevated dATP levels. The G_1/S boundary effect will probably be seen in other cell lines and with other nucleosides.

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