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DNA Fragmentation and Cytotoxicity from Increased Cellular Deoxyuridylate†

Holly A. Ingraham, Lisa Dickey, and Mehran Goulian*
Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, California 92093
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ABSTRACT: Previous results from this laboratory have shown that thymidylate deprivation results in dramatic elevation of intracellular dUTP and incorporation of dUMP into DNA. The goal of the present studies was to determine whether the latter changes may play a part in the associated cytotoxicity ("thymineless death"), which is ordinarily assumed to be a direct result of reduced intracellular dTTP. The approach used here was to increase intracellular dUTP without allowing dTTP to diminish and observe the effects on cell viability. dUMP pools were expanded by exposure of cells to deoxyuridine [in cell growth medium containing hypoxanthine, methotrexate, and thymidine (HAT medium)], resulting in accumulation of dUTP to levels that approached those of dTTP, which were at, or higher than, the levels in untreated cells. In conjunction with this the cells became nonviable, and newly synthesized DNA was fragmented, both of which occur with thymidylate depletion and, we assume, result from the active process of excision repair at the many uracil-containing sites in DNA. The results indicate that, although the relative importance of low dTTP remains unknown, elevated dUTP can account for the cytotoxicity caused by thymidine starvation. Most of the "dTTP" measured by the DNA polymerase assay in cells treated with methotrexate (MTX) (plus purine supplement) was, in fact, dUTP, which may explain some previous observations of only modest depression of dTTP in cells treated with MTX or similarly acting drugs.

Limitation of thymidine, caused either indirectly through the action of the dihydrofolate reductase inhibitor MTX[1] (with purine supplementation) or directly by inhibition of thymidylate synthetase by FdUrd, is accompanied by rapid loss of cell viability. These drug-induced equivalents of "thymineless death" have ordinarily been assumed to result from insufficient intracellular concentrations of dTTP although the mechanism

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* Author to whom correspondence should be addressed.

[1] Abbreviations: dThd, thymidine; dUrd, deoxyuridine; dUTPase, deoxyuridine triphosphatase; EDTA, ethylenediaminetetraacetic acid; FdUrd, 5-fluorodeoxyuridine; HAT, cell growth medium containing hypoxanthine, methotrexate, and thymidine; HPLC, high-pressure liquid chromatography; Hx, hypoxanthine; MTX, methotrexate; P_i, inorganic phosphate; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; Ura, uracil.

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by which this causes cell death is not understood [reviewed in Barclay et al. (1963)]. Recent observations on the consequences of alterations in intracellular dUrd nucleotide metabolism that accompany the fall in dTTP have offered the possibility of explaining some of the toxic changes of thymine limitation including the fragmentation of DNA that results. There is a very large increase in intracellular dUMP, of sufficient magnitude to result in accumulation of dUTP to levels that approach the reduced level of dTTP (Goulian et al., 1980a). As a result, dUMP is incorporated into DNA, activating the normal mechanism for removal of dUMP from DNA, a process of excision/repair that should result in multiple transient interruptions in the DNA (Goulian et al., 1980b; Sedwick et al., 1981). We report here results of experiments intended to test whether the resulting lesions in DNA may play a part in the toxic effects of methotrexate.

Experimental Procedures

Cell Growth, Labeling, and Acid Extraction. The hamster lung fibroblast line CCL39 was obtained from Dr. Gerard Buttin and grown in modified Eagle's medium with 10% fetal calf serum (de Saint Vincent & Buttin, 1979). The standard medium, both with and without MTX, contained a purine source, hypoxanthine (100 μM), unless stated otherwise. HAT medium contained 100 μM hypoxanthine, 10 μM MTX, and 5 μM dThd. No effect was observed on rate of growth or viability of cells grown in HAT medium as compared to normal medium. Cells were grown in 9-cm plastic dishes; 10^6 cells were plated in standard medium 18 h prior to the beginning of an experiment. Most of the experiments were carried out with undialyzed serum; no difference was seen in the results with dialyzed serum. All experiments, in HAT medium conditions, in MTX, or in standard medium (controls), were for 24 h; cell counts were obtained on duplicate cultures. Cell volume was determined with the Coulter Counter, Model ZBi.

To label cells with ^{32}P, after 22 h under experimental conditions (HAT ± dUrd, MTX, or standard medium control), the medium was replaced with the same medium as in the previous 22 h except for omission of phosphate (when dialyzed serum was used, one-tenth the amount of phosphate in standard medium was added). After 1 h in low-phosphate medium, 10 μCi/mL [^{32}P]P_i was added and incubation continued for 1 h. Total incorporation into DNA in normal medium was approximately 130000 cpm (per 9-cm dish).

To prelabel DNA, cells were grown in [2-^{14}C]dThd (0.1 μCi/mL, 56 mCi/mmol) for 16 h in standard medium, followed by an additional 1 h in standard medium without [^{14}C]dThd, before the experiment was begun.

To measure viability, cells were rinsed with phosphate-buffered saline, detached with trypsin/EDTA, counted, and plated at several dilutions in fresh standard medium. Individual clones were visible by 5 days; at 6 days they were fixed with 95% ethanol, stained with 1% crystal violet in 20% methanol, and counted. Cloning efficiency of the cells from cultures grown in standard medium (or HAT) was 40–60%.

To prepare extracts for nucleotide measurements, cells were rapidly rinsed (2 times) with ice-cold 0.02 M potassium phosphate, pH 7.6/0.15 M NaCl and then immediately extracted (3 times) with 5% perchloric acid (0 °C), the pooled supernatant of which was neutralized with KOH without delay. After removal of $KClO_4$ the extracts were concentrated and stored at −20 °C.

Separate (duplicate) plates were used for the ^{32}P labeling (sucrose gradient sedimentation velocity analysis of DNA), viability (and cell volume), and cell nucleotide pool extractions.

Nucleotide Analysis. A form of the DNA polymerase assay (Solter & Handschumacher, 1969; Skoog, 1970) was used to measure dNTPs. Poly(dA-dT) was the template/primer used for dATP and dTTP, and poly(dI-dC) was used for dGTP. The incubations (37 °C) were carried out in 20 μL containing 50 mM Tris·HCl, pH 7.6, 50 mM potassium acetate, 5 mM magnesium acetate, 0.1 mg/mL bovine serum albumin, 0.01% Nonidet P-40, 10% glycerol, and 0.001 unit of *Escherichia coli* DNA polymerase I (Jovin et al., 1969). It contained, in addition, the following: in the assay for dTTP, 150 ng of poly(dA-dT) and 0.5 μM [α-^{32}P]dATP (20 nCi/pmol); for dATP, 150 ng of poly(dA-dT) and 1 μM [*methyl*-^{3}H]dTTP (50 nCi/pmol); for dGTP, 40 ng of poly(dI-dC) and 0.5 μM [α-^{32}P]dCTP (50 nCi/pmol). After a 30-min incubation at 37 °C, 0.5 mL of a mixture containing 5 mM EDTA, 0.1 M potassium pyrophosphate, and 50 μg of carrier herring sperm DNA was added, followed by 0.5 mL of 2 M HCl. After 5 min at 0 °C the precipitated DNA was collected on glass fiber filters (Whatman GF/C), washed with 0.01 M HCl and ethanol, dried, and counted in a toluene/PPO/POPOP mixture. The lower limit of sensitivity (3 times the blank) was 0.1 pmol for dGTP and dTTP and 0.03 pmol for dATP, and linear ranges were up to at least 2 pmol for all three. Authentic dNTPs, alone or mixed with cell extracts, were recovered completely. Different amounts of extract were always tested to confirm a linear response. The apparent inhibitory effect of some extracts appeared to be due primarily to dilution of the labeled dNTP in the assay by unlabeled dNTP in the extract. This problem was avoided for assays of dTTP or dCTP by incubating the extract at 37 °C for 1 h in 0.1 M HCl (to destroy dATP or dGTP) (followed by neutralization), and for dGTP and dATP by using higher concentrations of labeled dNTP in the assay mixture, and was minimized, in general, by using only small amounts of the extracts.

A modified procedure was used for the dCTP values reported here. It was carried out in 20 μL containing 50 mM Tris·HCl, pH 7.6, 50 mM potassium acetate, 5 mM magnesium acetate, 0.1 mg/mL bovine serum albumin, 0.01% Nonidet P-40, 10% glycerol, 200 ng of primed single-stranded circular DNA template [prepared by heating a mixture containing 50 mM Tris, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 400 μg/mL bacteriophage M13 mp8 DNA, and 1.7 μg/mL 15-nucleotide (complementary) primer (New England Biolabs) to 100 °C for 2 min, followed by incubation at 60 °C for 20 min], dGTP and dTTP each at 50 μM, 20 μM [α-^{32}P]dATP (2.5 nCi/pmol), extract, and 0.1 unit of *E. coli* DNA polymerase I "large fragment" ("Klenow enzyme") (BRL). After incubation for 30 min at 37 °C it was processed as described for the other DNA polymerase assays of dNTP. The useful (linear) range was 1–20 pmol of dCTP.

dUTP was measured by determining the difference in the assays for dTTP, with and without pretreatment with dUTPase. Extract (equivalent to 10^5 cells) was incubated (30 min, 37 °C) in a mixture (20 μL) of 20 mM Tris·HCl, pH 7.6, 20 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 0.5 mg/mL bovine serum albumin, and 0.05 unit of dUTPase purified from human lymphoblastoid cells (Ingraham & Goulian, 1982); a control contained enzyme diluent in place of dUTPase. Appropriate dilutions were directly assayed as for dTTP by using DNA polymerase. Standard curves for dUTP and dTTP without dUTPase were indistinguishable; with dUTPase treatment dTTP values did not change whereas dUTP fell to background. (The amounts of dUTPase used were sufficient to eliminate at least 10 times the largest amount used in the assay standard curve.) The sensitivity of this procedure is

Table I: Effects of dUrd (in HAT) and MTX on Cell Viability and Cellular Nucleotides[a]

growth conditions	cell no.[b] × 10^{-6}	rel viability (%)	dUMP[d]	dUTP[d]	dTTP[d]	dATP[d]	dGTP[d]	dCTP[d]	UTP[d]	ATP[d]	GTP[d]	CTP[d]
std medium (+Hx)	8.0	(100)	5.8	NM[c]	54	50	9.2	86	1620	5400	1160	880
(std medium (-Hx)	7.7	(100)	5.9	NM	53	39	7.5	90	2800	3700	620	1280
HAT	7.6	(100)	320	NM	30	78	3.5	132	1530	5800	1000	880
HAT + 0.1 mM dUrd	7.2	83–100	790	NM	28	72	3.7	176	1620	6100	1000	900
HAT + 1 mM dUrd	5.2	57–80	1920	17	36	80	5.8	380	2500	8100	1590	1380
HAT + 10 mM dUrd	1.8	0–9	4800	139	147	131	15.7	260	4800	14600	3600	3200
MTX (+Hx)	2.0	0–2	2600	15	1.7	250	2.9	320	4400	19100	3700	3100
MTX (-Hx)	1.9	0–2	174	1.1	4.7	35	1.8	108	1110	1200	560	770

[a] See Experimental Procedures for details; the values given are the averages of three to eight experiments. [b] Cell number per 9-cm culture dish at end of a 24-h experimental period (average of seven experiments). [c] NM, not measurable, is less than 20% of the value for dTTP (see Experimental Procedures). [d] Values are given in pmol/10^6 cells.

limited by the amount of dTTP present in the same extract; it was not considered reliable for values of dUTP that were less than one-fifth those for dTTP. A similar method has been described by Williams et al. (1979).

dUMP was determined by HPLC. For higher values (>100 pmol/10^6 cells) it was measured directly on the extract by using an anion-exchange column (Whatman SAX) with 0.01 M ammonium phosphate, pH 3.25. For lower values (e.g., cells in normal medium) the acid extract was treated with IO_4^- (Garrett & Santi, 1979) (0.1 mL of IO_4^- incubation mixture per 5 × 10^6 cells), diluted (100-fold with water), applied to a DEAE-Sephadex A-25 (HCO_3^-) column (0.5-mL bed volume per 25 mL diluted extract), and washed with 20 column volumes of 0.02 M triethylammonium bicarbonate. It was eluted with 0.125 M triethylammonium bicarbonate and, after removal of the solvent, either analyzed on the anion-exchange column (above) or dephosphorylated with phosphatase and analyzed by HPLC on a reverse-phase column (RP-18) (Goulian et al., 1980a). Recovery of a tracer amount of [^{3}H]dUMP added with the acid extraction was used to correct for losses.

rNTPs were measured by HPLC with an anion-exchange column (Whatman SAX) using 0.4 M ammonium phosphate, pH 3.6.

DNA Extraction and Analysis. At the completion of the 24-h experimental period, during the last hour of which the cells were labeled with [^{32}P]P_i (see above), the cells were rinsed 3 times with 0.02 M potassium phosphate, pH 7.6/0.15 M NaCl and lysed with 1.5 mL of a mixture containing 0.05 M Tris·HCl, pH 8, 0.01 M Na_3EDTA, 1% sodium dodecyl sulfate, and 1 mg/mL Pronase (Calbiochem). After 2 h at 37 °C the lysate was gently poured into a 10-mL Beckman heavy-wall ultracentrifuge tube and incubated an additional 15 h at 37 °C. A 2.5-mL aliquot of 5.4 M lithium trichloroacetate was added and the mixture slowly rotated at room temperature for 90 min. The resulting homogeneous mixture was heated at 60 °C for 30 min (to denature the DNA), and after being cooled to room temperature, 4.5 mL of 50 mM Tris·HCl, pH 8, 10 mM Na_3EDTA, and 1% sodium dodecyl sulfate was added; then, it was mixed by slow rotation for 60 min. The mixture was then centrifuged for 1 h at 30000 rpm, 20 °C, in the Beckman type 65 rotor to pellet high molecular weight DNA. The "low molecular weight" DNA fraction in the supernatant was precipitated with 2.5 volumes of ethanol (-20 °C overnight), recovered by centrifugation, redissolved in 0.5 mL of 0.05 M NaOH and 0.001 M EDTA, and after 1 h at 37 °C (to digest RNA), neutralized with 2 M acetic acid. Carrier yeast RNA (200 μg/mL) and KCl (to 0.5 M) were added, it was reprecipitated with 2.5 volumes of ethanol, and after centrifugation, it was dissolved in 0.25 mL of 0.01 M Tricine·HCl [*N*-[tris(hydroxymethyl)methyl]glycine hydrochloride], pH 8, and 0.001 M EDTA and stored at 4 °C. Aliquots (0.015–0.1 mL) were made 0.3 M in NaOH and, after addition of ^{3}H-labeled *E. coli* bacteriophage fd DNA marker, were layered over 3.8-mL sucrose gradients (5–20%) in 0.3 M NaOH, 0.7 M NaCl, and 0.01 M EDTA, over a 0.2-mL shelf of the 20% sucrose solution saturated with CsCl. The tubes were centrifuged in the Beckman SW 60 rotor for 6 h at 45000 rpm (20 °C); fractions were collected from above, acid-washed, and counted on glass fiber discs.

Results

In initial attempts to increase intracellular dUrd nucleotide pools, dUrd was simply added to the standard growth medium. Even at concentrations as high as 10 mM, dUrd had no detectable effect on growth rate or viability (plating efficiency), and intracellular dUMP measurements showed only a small increase over the normal range (to 30–40 pmol/10^6 cells). Intracellular dTTP also was elevated, to levels of 400–700 pmol/10^6 cells. Further expansion of the dUMP pool appears to have been limited, and this may have been by inhibition of dThd kinase by the increased concentration of dTTP (see Discussion).

In contrast, when conversion of deoxyuridylate to deoxythymidylate was blocked by MTX in cells provided with an exogenous source of dThd and purine i.e., "HAT" medium, dUrd added to the growth medium had a pronounced effect on intracellular dUrd nucleotide pools (Table I). At the higher levels of dUrd in the medium, intracellular dUMP exceeded even the very high concentrations seen with MTX (+Hx) (Table I; Goulian et al., 1980a). Correlated with the increase of dUMP in the cells exposed to HAT + dUrd there was inhibition of cell growth and loss of viability that, at the highest concentration of dUrd in the medium, amounted to >90% cell death (Table I). Smaller increases of intracellular dUMP were also seen with HAT medium without dUrd, possibly reflecting a somewhat lower than normal level of dTTP in these cells. dTTP in the cells in HAT medium with the highest concentration of dUrd (10 mM) was actually greater than in untreated cells. At the higher concentrations of dUrd in the medium there were easily measurable levels of dUTP that paralleled the increase in dUMP, and at the highest concentration of dUrd (10 mM) dUTP equaled the level of dTTP.

In cells exposed to MTX (+Hx) for the same period of time dUTP was much lower (by approximately one-tenth) than in HAT + 10 mM dUrd; however, dTTP was proportionately even lower, resulting in a ratio dUTP/dTTP of 9/1. There was almost complete loss of viability (>98%) of cells treated with MTX (+Hx).

To test the effect on DNA single-strand length, a method was used that had been developed previously for a similar

Table II: Cellular Nucleotide Pools Related to Cell Volume[a]

growth conditions	cell vol (μm^3)	dUMP[b]	dUTP[b]	dTTP[b]	dATP[b]	dGTP[b]	dCTP[b]	rUTP[b]	rATP[b]	rGTP[b]	rCTP[b]
std medium (+Hx)	1230	4.7	NM	44	41	7.5	70	1320	4400	940	710
std medium (-Hx)	1340	4.4	NM	39	29	5.6	67	2100	2800	460	960
HAT	1440	220	NM	21	54	2.4	92	1070	4100	700	610
HAT + 0.1 mM dUrd	1490	530	NM	19	48	2.5	118	1090	4100	740	610
HAT + 1 mM dUrd	1990	970	8.6	18	40	2.9	191	1260	4100	800	690
HAT + 10 mM dUrd	2600	1850	54	57	51	6.1	102	1870	5600	1390	1240
MTX (+Hx)	3000	860	5.0	0.6	83	1.0	105	1450	6300	1220	1010
MTX (-Hx)	1920	91	0.6	2.4	18	0.9	56	580	620	290	400

[a] Values of Table I are expressed here per unit cell volume. If distribution of nucleotides is uniform, the values would approximate concentration (μM). [b] Values are given in pmol/μL.

purpose (Grafstrom et al., 1978; see Experimental Procedures). By this procedure the highest molecular weight chromosomal DNA was first removed to minimize anomalies due to viscosity and gel formation; the remaining low molecular weight fraction of DNA was analyzed on alkaline sucrose gradients. The results of the analyses on newly synthesized DNA (1-h labeling with ^{32}P) from cells grown in standard medium or in HAT (or in HAT + 0.1 mM dUrd) were not distinguishable; the peak of DNA size distribution was at the bottom of the gradient, and most of the DNA was in the lower one-fourth of the gradient (>20 S) (Figure 1A,C,D). In contrast, with MTX (+Hx), the size of the labeled DNA was very small, almost all of it in the top one-fourth of the gradient (<7 S) (Figure 1B). With HAT + 1 mM dUrd there was a slight shift toward smaller sizes of DNA (Figure 1E) whereas with HAT + 10 mM dUrd the reduction is pronounced, resembling the results with MTX (+Hx) (Figure 1F).

When similar analyses were carried out on the DNA of cells prelabeled with [^{14}C]dThd, in all cases, including standard medium, HAT, HAT + 10 mM dUrd, and MTX (+Hx), the pattern of the prelabel was the same (not shown) and was similar to what was seen with the ^{32}P label in Figure 1A,C. We infer from this that the changes detected by the procedure used for the experiments shown in Figure 1 were primarily at or near replication sites.

Not surprisingly, cell number increase correlated with viability (Table I). The figures for incorporation of ^{32}P with DNA generally reflected cell growth rate and viability; however, it is of interest that both with MTX (+Hx) and with HAT + 10 mM dUrd considerable incorporation of ^{32}P into DNA remained: approximately 15% and 35%, respectively, of the amount for standard medium (see Discussion).

Compared to cells in standard medium, there were approximately 3-fold and 5-fold increases in dATP in HAT + mM dUrd and MTX (+Hx), respectively. dCTP also increased 3–4-fold for the higher levels of dUrd (in HAT) and MTX (+Hx), compared to standard medium. In spite of the purine supplement dGTP levels were generally lower than normal in cells in HAT (±0.1 mM dUrd) and MTX (+Hx).

The rNTPs showed increases up to 3–4 times normal in the most severely inhibited cells, i.e., in HAT + 10 mM dUrd and in MTX (+ Hx) (Table I).

Omission of the purine supplement (Hx) with standard medium had only minimal effects—slightly lower purine rNTPs and dNTPs, slightly higher pyrimidine rNTPs, and no effect on pyrimidine dNTPs. In contrast, omission of the purine supplement profoundly altered the effects of MTX on nucleotide pools. Compared to MTX with Hx, MTX without Hx caused less increase in dUMP and dUTP and less fall in dTTP (although the latter was still one-tenth of normal), to give a ratio dUTP/dTTP of ~1/4 (instead of 9/1 as in the presence of Hx). Also, instead of the marked *increases* (compared to standard medium) in the other nucleotides (with

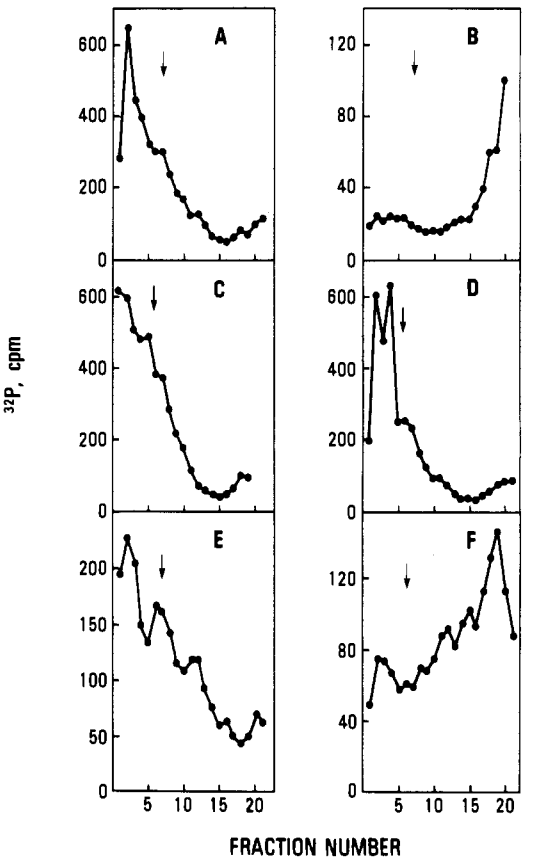

FIGURE 1: Alkaline sedimentation velocity of DNA from cells treated with dUrd (in HAT medium) or MTX. The low molecular weight fraction of DNA was prepared from ^{32}P-labeled cells, and samples were analyzed on alkaline sucrose gradients (Experimental Procedures): (A) standard medium; (B) MTX (+Hx); (C) HAT medium; (D) HAT + 0.1 mM dUrd; (E) HAT + 1 mM Urd; (F) HAT + 10 mM dUrd. Direction of sedimentation is to the left, and fractions, although collected from the top, are numbered by their distance from the bottom of the gradient. Arrows indicate the position of the internal 20S marker, ^{3}H-labeled fd DNA.

the exception of dGTP) that occurred with MTX + Hx, MTX without the purine supplement caused *reductions* except for dCTP and CTP (which remained the same as with standard medium).

The values for intracellular nucleotides are expressed in Table I as the amount per cell (pmol/10^6 cells). However, there was a noticeable increase in cell size (microscopically) with dUrd (10 mM) in HAT medium and with MTX (+Hx), which, presumably, reflects unbalanced growth, as first described for thymine-deprived prokaryote cells (Barclay et al., 1963; Cohen & Barner, 1954). If it is assumed that intracellular distribution of nucleotides is uniform (see Discussion), the volume differences significantly affect comparisons of intracellular nucleotides in treated and untreated cells. When the values are expressed per unit cell volume (Table II), the

large increases in dATP, dCTP, and rNTPs with HAT + dUrd and MTX + Hx largely disappear, and the amounts of dTTP and dGTP per unit cell volume with MTX + Hx (compared to standard medium) (Table II) are even lower than the amounts per cell (Table I).

Discussion

In the presence of HAT medium containing dUrd, intracellular dUrd nucleotide concentration increased sufficiently to cause accumulation of dUTP to extraordinarily high levels in spite of the dUTPase mechanism. This allowed measurements of dUTP by an assay that was much less sensitive (although much more convenient) than the one used previously (Goulian et al., 1980a). At the highest levels of dUrd in the medium (HAT + 10 mM dUrd), dUTP was almost 10 times the level in cells treated with MTX (+Hx). Although dTTP concentration was also somewhat increased with HAT + 10 mM dUrd, dUTP was sufficiently elevated to give a ratio dUTP/dTTP of approximately 1. The single-strand length of newly synthesized DNA from these cells was markedly reduced, and most of the cells died. dUTP was also easily measurable at the intermediate amount of dUrd (HAT + 1 mM dUrd), but here the ratio of dUTP/dTTP was lower and was associated with only small effects on viability and DNA size.

The results with HAT + 10 mM dUrd may represent an in vivo counterpart to an earlier model of excessive dUMP incorporation into nuclei in vitro which resulted in irreversible fragmentation of newly synthesized DNA (Grafstrom et al., 1978). The effects of the intermediate level of dUrd (1 mM) may correspond more closely to observations with the original dUTPase-deficient mutants of *E. coli* (*dut* or *sof*), in which fragmentation of the DNA had little or no effect on viability (Tye et al., 1977, 1978). The ratio of dUTP/dTTP ($\sim$1/2) in the cells in HAT + 1 mM dUrd implies substantial incorporation of dUMP (Goulian et al., 1980b) and consequently many repair sites/interruptions. The fact that DNA size is affected only minimally may indicate that, as in the case of *E. coli dut* mutants, the repair capacity under these conditions was not greatly stressed and/or the particular method used here to assess DNA strand size (1-h labeling) allowed sufficient time for substantial completion of DNA repair.

The nature of the lesion that leads to the death of cells with high ratios of dUTP/dTTP remains unclear. The lethal event may be related to the probability that gap filling of a dUMP removal/repair site may itself result in reinsertion of dUMP, initiating further removal of dUMP, leading to a futile cyclic process of reinsertion and removal (Goulian et al., 1980a,b). Depending on the ratio dUTP/dTTP as well as several presently inevaluable factors, e.g., average repair patch size, activities of individual components of the repair mechanism, and availability of substrates (see below), gaps at repair sites may persist or even increase in size. Progressively increasing gap size seems very likely when dUTP is considerably greater than dTTP, as in this study, since there is a high probability that more than one dUMP will be incorporated in the repair following excision of a single dUMP. Large single-stranded gaps may be more susceptible to nuclease attack (resulting in double-stranded breaks) or other forms of structure disruption that are difficult to repair (Hanawalt et al., 1979). At present this model remains purely speculative although certain features of it may be testable. Of particular interest is the fact that in prokaryotes very severely deficient in dUTPase there are pronounced effects on growth and viability that are directly related to the removal and repair of uracil-containing sites in DNA (Warner et al., 1981; Hochhauser & Weiss, 1978).

What can be said about the relationship between the effects demonstrated here of a high ratio of intracellular dUTP/dTTP with normal dTTP and the cytotoxicity due to thymidylate deprivation? Both are capable of rapidly causing cell death. Both result in fragmentation of DNA. DNA fragmentation associated with thymidylate limitation has long been observed in both prokaryotes and eukaryotes (Barclay et al., 1963; Salzman & Thoren, 1973; Perlman & Huberman, 1977; Cheng & Nakayama, 1983) and has been reported to include double-strand breaks (Ayusawa et al., 1983; Li & Kaminskas, 1984). It appears likely that the enhancement of spontaneous chromosome breakage that results from thymidylate limitation (produced by various nutritional or pharmacological methods) (Jacky et al., 1982) is a gross reflection of the same process.

It must be assumed that, whatever the detailed mechanism for the cytotoxic effects of dUMP insertion/removal (and repair) seen with HAT + 10 mM dUrd, a similar process occurs in cells deficient in thymidylate, whether caused by MTX (+Hx) (Goulian et al., 1980a,b) or FdUrd (Ingraham et al., 1982), or in thymidine-requiring mutants deprived of an exogenous source (Ayusawa et al., 1983). The high residual incorporation of ^{32}P into DNA with either HAT + 10 mM dUrd or MTX (+Hx) (see Results) probably reflects, at least in part, the turnover that accompanies repetitive insertion and removal of dUMP.

The results of this study demonstrate that dUMP incorporation/removal *could* account for DNA fragmentation and cell death resulting from MTX-induced deficiency of thymidylate. Nevertheless, they do not establish how important these effects are in causing the cytotoxicity that results from MTX, since the consequences of a low level of dTTP, by itself (i.e., without increase in dUTP), are not known. It is pertinent to note that, in bacteria, the toxicity that results from thymine starvation is reduced when the uracil removal mechanism is impaired by mutation in the gene for Ura–DNA glycosylase (Makino & Munakata, 1978; Lindahl, 1979). This invites comparison to the mitigating effect of a similar mutation on the growth inhibition and loss of viability that occur with severe loss of dUTPase (Warner et al., 1981; Hochhauser & Weiss, 1978). Thus, in prokaryotes, the toxicity of both depressed thymidylate and elevated cellular dUTP is associated with *removal of uracil from DNA.*

Levels of dTTP that are not markedly depressed have been observed with MTX toxicity (Tattersall & Harrap, 1973; Roberts & Peck, 1981; Bestwick et al., 1982; Taylor et al., 1982). The results here suggest one explanation: significant amounts of dUTP may be present, and in the usual assay for dTTP (using DNA polymerase) the concentration of dTTP is the sum of those of dUTP and dTTP (see Experimental Procedures). It should be noted, however, that in the results reported here the discrepancy between measured and actual amounts of dTTP was pronounced only when the purine supplement was present with MTX.

The other dNTPs and rNTPs were measured primarily to detect unsuspected perturbation(s) that may account for the observed effects of expanded dUrd nucleotide pools. Stimulation of ribonucleotide reductase by depressed dTTP has been identified as a factor in expansion of the dUrd nucleotide pool with inhibition of thymidylate synthetase (in addition to "de-inhibition" of dCMP deaminase) (Jackson, 1978). The results here indicate an additional reason for enhanced activity of ribonucleotide reductase on both UDP and CDP, the stimulation by low dGTP (Thelander & Reichard, 1979).

The apparent large increases in rNTPs with MTX and HAT + dUrd (expressed per cell number; Table I) may be related,

at least in part, to increased cell size that occurs under these conditions; intracellular *concentrations* may not be greatly affected. The effects on rNTPs are not an artifact of purine supplementation since Hx had only a slight effect on cells in the absence of MTX and entirely similar values result with FdUrd treatment (without purine supplement), which causes similar cell-size increases (unpublished experiments). We assume also that the changes in cell size account for most of the apparent increases in dATP and dCTP with MTX (+Hx) and high dUrd as well; again, similar changes occur with FdUrd.

Low dGTP has been observed previously in MTX-treated cells, even with purine supplementation (Taylor et al., 1982). The role of ribonucleotide reductase is not clear here, since low dTTP and dGTP would tend to stimulate conversion of GDP to dGDP (Thelander & Reichard, 1977). It has been suggested that low dGTP may play a part in the toxicity of MTX (Bestwick et al., 1982).

There are marked differences in nucleotide pools between cells treated with MTX in the presence and absence of purine supplementation (see Results; Taylor et al., 1982). This includes the expected ability of Hx to prevent the reductions in purine dNTP and rNTP pools by MTX (with the exception of the fall in dGTP which occurs nevertheless). Of particular interest is the profound difference in the ratio dUTP/dTTP between MTX-treated cells that are permitted to deplete purine pools vs. those that are not (0.25 vs. 9). This suggests the possibility that the reported enhancement of toxicity from MTX with purine supplementation (Taylor et al., 1982) may be mediated by augmented incorporation of dUMP into DNA. Without additional information one can only speculate on how these observations, the results in this paper as well as those of Taylor et al. (1982), both of which relied on cultured cells, relate to the effects of the drug in intact animals; factors such as species, cell/tissue, growth state/differentiation, and nutritional factors may affect this. However, the fact that dThd alone "rescues" humans from the toxic effects of MTX (Ensminger & Frei, 1977) (implying dietary or tissue turnover for other "salvage" sources of purines) suggests that clinical responses may resemble more closely the purine-supplemented cell culture model. If so, it is likely that dUrd nucleotide pool expansion plays a significant part in the toxic effects of MTX under clinical circumstances as well.

Registry No. dUrd, 951-78-0; dUTP, 1173-82-6; dUMP, 964-26-1; thymine, 65-71-4; methotrexate, 59-05-2.

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