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RAPID CHANGES IN DEOXYNUCLEOSIDE TRIPHOSPHATE POOLS IN MAMMALIAN CELLS TREATED WITH MUTAGENS

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SUMMARY: In this communication we describe the rapid increase in cellular deoxynucleoside triphosphate (dNTP) concentrations in Chinese Hamster cell line V79 after exposure to known mutagens. With this cell line an expansion of dATP and dTTP pools was detected; changes in dCTP were not large; changes in dGTP were either not significant or too low to quantitate. This situation may reflect the existence of imbalances in dNTP pools at the DNA replication fork. The expansion of dATP and dTTP pools occurred within 2 to 4 hours after exposure of cultured cells to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Ultraviolet light (UV), mitomycin C, and cytosine arabinoside also caused similar dNTP pool changes.

INTRODUCTION: Replication of DNA requires four deoxynucleoside triphosphates (1). In mammalian cells these precursors are derived mainly from the reduction of the corresponding ribonucleoside diphosphates catalyzed by the enzyme ribonucleotide reductase (2). The small steady-state concentrations of the dNTPs and the multiple regulatory effectors of ribonucleotide reductase indicates that the biosynthesis of dNTPs is under complex controls (2). Currently available evidence indicates that this may be necessary to ensure error-free DNA replication. The dependency of the fidelity of DNA synthesis in vitro on the relative concentrations of dNTPs suggests that pool imbalances could have mutagenic consequences (3).

MATERIALS AND METHODS: Exponentially growing V79 cells were plated approximately 30 hr before the experiment. Growth medium was minimal Eagle's medium supplemented with 10% heat-inactivated dialyzed fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in a humidified incubator in 5% CO_2 atmosphere. MNNG at a final concentration of 5 μM was added at t = -1 hr. At the end of 1 hr incubation at 37°C, the medium was replaced with fresh medium without MNNG. For ultraviolet treatment, the medium was removed and cells were covered with sterile phosphate-buffered saline (PBS). After ultraviolet irradiation (1.5 $J \cdot m^{-2}$/sec), PBS was removed and fresh medium was added to these cultures. Cell extracts for dNTP pool estimation were prepared by

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aspirating the medium, quickly washing with ice-cold PBS, and scraping with 60% ice-cold methanol. These cells were stored overnight at -20°C. Cell debris was removed by centrifugation, and the 60% methanol supernatant was made 0.5 N $HClO_4$ and kept at 4°C for 2 hr (4). After clearing the solution by centrifugation, these extracts were neutralized by adding KOH, and the precipitate was removed by centrifugation. Pool measurements were carried out using E. coli DNA polymerase I assay with poly(dA-dT) and poly(dG-dC), as described by Skoog et al. (5).

RESULTS: Exponentially growing Chinese hamster V79 cells have small but measurable quantities of all four dNTPs. Their amounts per 10^6 cells are approximately: dATP-40 pmol, dTTP-43 pmol, dCTP-78 pmol, dGTP-4 pmol (Fig. 1, Control). The low concentration of dGTP is similar to that reported in cell lines including V79 (4,6,7). Treatment of V79 cells with 5.0 μM N-methyl-N'nitro-N-nitrosoguanidine (MNNG) results in a rapid increase in dATP and dTTP pools. In fact, the increase in pool sizes can be detected as early as 2 hours after the beginning of drug treatment (Figure 1). After MNNG treatment, the dATP pool increases for 4 hours and remains at the elevated level for as long as 8 hours, and returns to that of untreated cells by 16 hours. In three separate experiments, the increase in dTTP pool follows different kinetics compared to dATP; maximum levels are

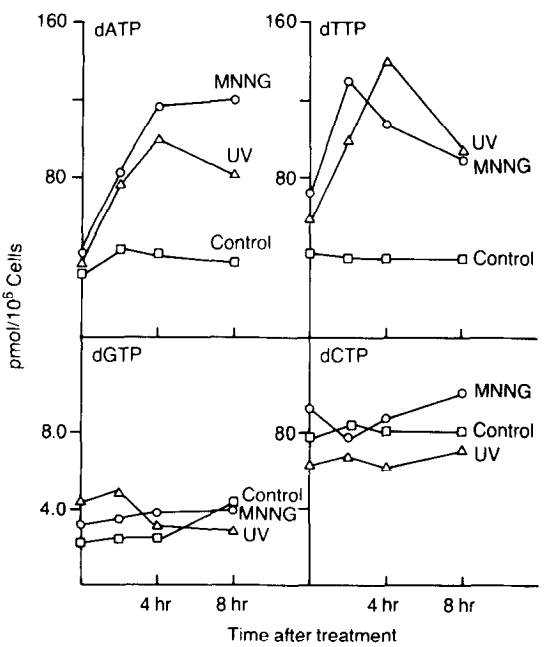

Figure 1. dNTP pool changes in exponentially growing V79 cells treated with mutagens. MNNG concentration was 5 μM and UV fluence was 1·5 $J \cdot m^{-2}$/sec.

attained 2 hours after addition of 5.0 μM MNNG. In five experiments with MNNG-treated cells, the maximum increase in dATP and dTTP pools ranged from 3-fold to 8.5-fold, and 1.6-fold to 4.5-fold, respectively. During the same time interval, dCTP and dGTP pools changed by less than 2-fold.

The concentrations of MNNG used in this study killed approximately 10% of the V79 cells as determined by clonogenic assay and resulted in a mutation frequency of 50×10^{-5} at the HGPRT locus (thioguanine resistance), compared to 1.6×10^{-6} spontaneous frequency (8,9). Thus the effect on dNTP pools occurs at concentrations of MNNG which allow relatively high survival. Since the pool perturbations seen in mutagen-treated V79 cells could simply be from some peculiarities in dNTP biosynthetic pathways in these cells, we examined the response of other cells. Four hours after treatment of low-passage human diploid fibroblasts (obtained from T. Norwood of this Department) and Syrian hamster BHK21 cells with 10 μM MNNG, the expansion of dATP and dTTP was observed.

A different class of mutagen, ultraviolet irradiation, which is known to terminate chain elongation, also increases dATP and dTTP pools (10,11). With V79 cells, the kinetics of pool expansion after ultraviolet irradiation appears to differ from that observed with MNNG-treated cells. As seen in Figure 1, both dATP and dTTP pools reach a maximum at 4 hours after ultraviolet treatment. In ultraviolet-treated cells, the levels of dGTP and dCTP did not increase significantly. The doses of ultraviolet light used in this study have also been shown by Stone-Wolff and Rossman to cause substantial mutagenesis at the Oua^r locus in V79 cells (12). Treatment of V79 cells with mitomycin C and cytosine arabinoside also caused elevation of dATP and dTTP pools. In these experiments dCTP levels were slightly decreased and dGTP levels were too low to be measured (Table I).

The effect of mutagen dose on deoxynucleoside triphosphate pools is seen in Figure 2. At low doses, the relative increase in dTTP pools was dependent on the amount of MNNG or the length of exposure to ultraviolet light. At high doses a maximum amount of pool imbalance was observed, which

Table I
Deoxynucleoside Triphosphates in V79 Cells
Treated with Cytosine Arabinoside and Mitomycin C

Treatment	Relative Change			
	dATP	dTTP	dGTP	dCTP
None	1.0	1.0	n.d.*	1.0
Cytosine arabinoside 0.5 μM for 8 hr	2.9	4.1	n.d.	0.72
Mitomycin C 0.5 μg/ml for 1 hr followed by 8 hr incubation	1.9	2.3	n.d.	0.83

* n.d. = not detectable.
V79 cells were plated 36 hr before the beginning of the experiment. Mitomycin C was added at t = -1 hr and removed at t = 0 hr. Fresh medium was then added to all plates; cytosine arabinoside was added at t = 0 and was continuously present. Extracts for dNTP pool measurement were prepared at t = 8 hr as described in Materials and Methods.

suggests a dose-saturation type of phenomenon. It should be pointed out, however, that pool-imbalance condition may persist for longer periods in cells treated with higher concentrations of mutagens.

The magnitude of pool expansion by MNNG reported in the paper is similar to that reported during the cell cycle (7), but the specificity appears to be different. Flow microfluorimetry indicates that the exponentially growing V79 cells used in this study have approximately 41% cells in G1, 43% in S phase, and about 16% cells in G_2 + M phase (Fig. 3A). This distribution is not significantly altered 4 hr after treatment with 5 μM MNNG (Fig. 3B). Furthermore, the increase in dATP and dTTP, and the lack of increase in dGTP and dCTP is not in accord with perturbation in cell

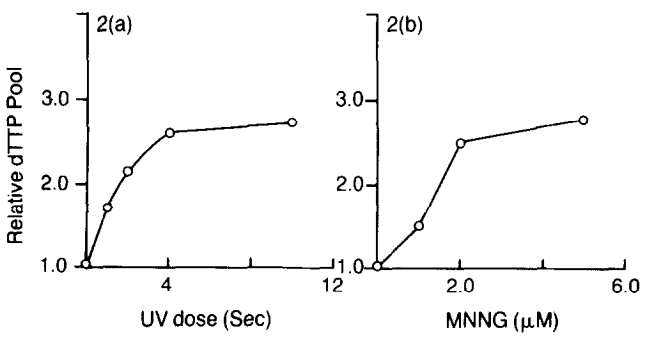

Figure 2. Exponentially growing V79 monolayers were treated with indicated doses of MNNG or ultraviolet irradiation as in Figure 1. Pool measurements were carried out at 4 hours after the treatment. UV fluence was 1·5 $J \cdot m^{-2}$/sec.

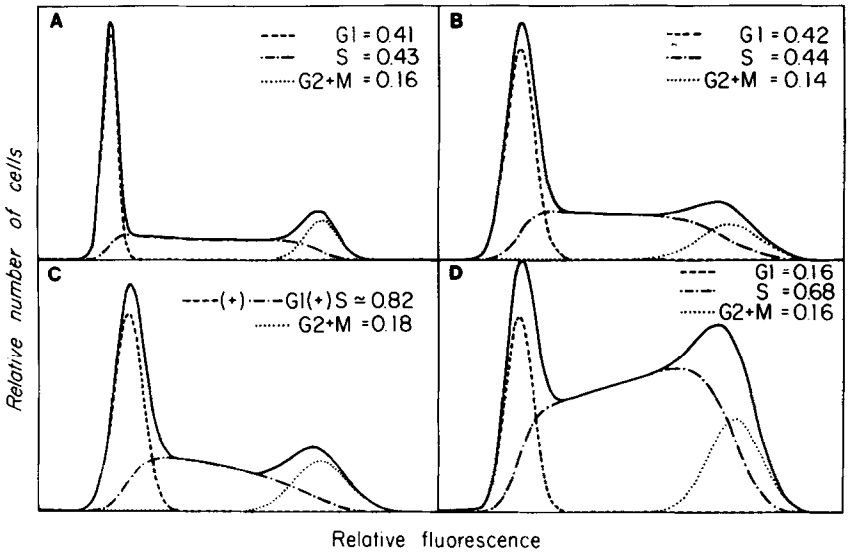

Figure 3. Flow microfluorimetric analysis of V79 cells. Exponentially growing cultures were subjected to the indicated treatments and prepared for flow cytometric analysis with 4,6-diamino-2-phenylindole and nonionic detergent, as described by Rabinovitch et al. (13). (A) control; (B) 5.0 μM MNNG for 1 hr followed by incubation for 4 hr.; (C) 0.4 μM cytosine arabinoside for 8 hr (continuous treatment); (D) 0.4 μg/ml Mitomycin C for 1 hr followed by 8 hr incubation in drug-free medium.

cycle (7,14). In synchronized cells, all four nucleotides increase at the onset of S phase. Mitomycin C and cytosine aribinoside treatment caused V79 cells to be blocked in S phase (Fig. 3C,3D), yet also resulted in similar nucleotide pool alterations.

DISCUSSION: These studies demonstrate that DNA damage by diverse agents alters cellular deoxynucleoside triphosphate concentrations in mammalian cells. These alterations are specific, occur rapidly, are reversible, and are not necessarily accompanied by cell-cycle changes. Presumably, changes in deoxynucleoside triphosphate concentrations are mediated by the rate-limiting enzyme, ribonucleotide reductase, which is subject to complex pathways of substrate and product regulation (2). This finding may be more general. In preliminary experiments we have also observed 5- to 10-fold changes in dATP and dTTP pools in *E. coli* after UV-irradiation; this increase seems to be recA- and umuC-independent (unpublished results). Studies with purified eucaryotic DNA polymerases indicate that the frequency of misincorporations is proportional to the relative concentration of the

four deoxynucleotide substrates (15,16). In cells, the relationship between the fidelity of DNA replication and pool size is likely to be more complex since DNA replication is probably carried out by a multienzyme complex, and noncomplementary nucleotides are subject to excision after incorporation, i.e., proofreading (17-19). Nevertheless, in vivo studies by Meuth et al. show that mutant cells with altered nucleotide pools exhibit a 5- to 50-fold enhancement in the rate of spontaneous mutagenesis (20). Analysis of certain mouse S-49 T lymphoma cells that exhibit increased mutagenesis contain alterations in nucleotide pools mediated by changes in ribonucleotide reductase (21). These studies demonstrate a relationship between changes in nucleotide pools and mutagenesis at multiple loci. Conversely, alteration in DNA polymerase affecting nucleotide binding can also be associated with mutagenesis. Liu et al. isolated an aphidicolin-resistant cell line which exhibited a mutator phenotype and contained a DNA polymerase-α resistant to aphidicolin in vitro (22). The apparent Km for dCTP with the mutant enzyme was 10-fold less than with the parent enzyme; no difference in the Km for dATP was detected.

Irrespective of mechanism, the effect of altering nucleotide pools by mutagens suggests that these alterations may be a significant component in mutagenesis by such agents. Moreover, altered nucleotide pools may provide an explanation for nontargeted mutagenesis that is nucleotide change not in immediate proximity to damaged nucleotides on DNA (23,24). Measurement of DNA repair based on incorporation of labeled thymidine or deoxyadenosine should be critically evaluated, since the endogenous pools of nucleotides are affected by DNA-damaging agents.

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