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Mechanism of 2-Aminopurine Mutagenesis in Mouse T-Lymphosarcoma Cells

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We investigated the mechanism of action of 2-aminopurine (Apur) in eucaryotic cells. By analogy with studies in procaryotic systems, the base analog is presumed to incorporate into DNA predominantly opposite T where, upon subsequent DNA replication, it can mispair with C, inducing an A:T → G:C transition. This model predicts that Apur-induced mutagenesis will be enhanced by factors that favor formation of Apur-C mispairs, e.g., high levels of dCTP or low levels of TTP. We describe the use of a mutant T-lymphosarcoma cell line, AraC-6-1, which has an abnormally high dCTP pool and a low TTP pool, to test this prediction. AraC-6-1 cells were three- to fivefold more mutable by Apur than their parental cell line, NSU-1. This enhanced mutability by Apur could not be explained by altered incorporation of ^{3}H-labeled Apur, by generally impaired ability to repair DNA damage, or by a direct effect of Apur on the endogenous deoxynucleotide pools. The addition of 10 μM thymidine to the growth medium of AraC-6-1 cells lowered their high dCTP pool (two- to threefold), raised the TTP pool (two- to threefold), and abolished their enhanced mutability by Apur. Further manipulation to produce an abnormally high TTP/dCTP ratio suppressed Apur-induced mutagenesis (8- to 10-fold) in both AraC-6-1 and NSU-1 cells. These observations support the hypothesis that Apur induces A:T → G:C transitions in mammalian cells by a mispairing mechanism.

The effects of the mutagenic purine analog 2-aminopurine (Apur) have been well studied in procaryotic systems including T4 bacteriophage and *Escherichia coli* (reviewed in reference 23). As an analog of adenine, Apur generally pairs with thymine, but mispairs with cytosine more frequently than does adenine. In procaryotes, Apur is usually incorporated into DNA opposite T and induces A:T → G:C transitions by mispairing with C in a subsequent round of DNA replication. Apur can also induce G:C → A:T transitions, although with a much lower frequency, by being erroneously incorporated into DNA opposite C and then pairing with T (6, 12, 14, 23). Apur has been reported to induce mutations in mammalian cells (2, 5). However, the mechanism of mutagenic action of Apur in eucaryotic systems is less clear. It has been suggested that in addition to its well-documented base-pairing ambiguity (10, 14, 23), Apur may be mutagenic by a second mechanism involving perturbation of deoxynucleotide triphosphate (dNTP) pools (15). Bromodeoxyuridine, the other well-studied mutagenic base analog, is also believed to exert twofold effects (15, 20). Perturbation of dNTP pools by Apur is presumed to result from inhibition of adenosine deaminase by the Apur metabolite Apur-deoxyribose (9), leading to accumulation of dATP, inhibition of ribonucleotide reductase by the high level of dATP (4, 22), and consequent depletion of the dGTP, dCTP, and TTP pools. It has recently been shown that similar perturbation of the endogenous dNTP pools by genetic means can be highly mutagenic in mammalian cells (21, 28).

The mispairing hypothesis predicts that Apur mutagenesis will be enhanced by factors that increase the ability of dCTP to compete with TTP (e.g., decreased levels of TTP, increased levels of dCTP, or a combination of both) opposite Apur templates. We recently described the isolation and characterization of a mutant mouse S49 T-lymphoma cell line (AraC-6-1) that lacks the enzyme dCMP deaminase and consequently accumulates high levels of dCTP and unusually low levels of TTP (28). We reasoned that this cell line would provide a useful system in which

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to test the mispairing hypothesis for Apur-induced mutagenesis.

We provide evidence that Apur is an effective mutagen in cultured mouse S49 T-lymphoma cells. We show that Apur does not significantly perturb endogenous dNTP pools. By pharmacologically manipulating the endogenous levels of dCTP and TTP and by comparing the mutability of AraC-6-1 and its parental line, NSU-1, we show that the level of Apur-induced mutagenesis is related to the levels of endogenous TTP and dCTP. Consistent with the model, low TTP with high dCTP enhanced Apur mutagenesis; high TTP suppressed Apur mutagenesis.

This report represents the first detailed study of the effects of Apur in mammalian cells and strongly supports the view that Apur induces mainly A:T → G:C transitions by a mispairing mechanism.

MATERIALS AND METHODS

Materials. We obtained Apur from Pfaltz and Bauer, Inc. (Stamford, Conn.), 6-^{3}H-labeled Apur from ICN, and 6-thioguanine from Burroughs-Wellcome Co. Sigma Chemical Co. supplied ouabain, dCTP, dATP, and dGTP; TTP was purchased from P-L Biochemicals, proteinase K was obtained from Merck & Co., and agarose was obtained from FMC Marine Colloids. All other reagents were of the highest grades commercially available.

Cell lines and culture techniques. The cell lines used in these experiments were S49 mouse lymphosarcoma cells (16) maintained in Dulbecco modified Eagle medium supplemented with 10% horse serum as described previously (11, 24). The isolation and characterization of the mutant lines NSU-1 (25) and AraC-6-1 (28) have been previously described. For the mutagenesis experiments, the NSU-1 and AraC-6-1 lines were recloned, and subclones were expanded to 1×10^7 to 2×10^7 cells and frozen in equal portions. One subclone from each line was used for the Apur mutagenesis experiments to avoid large variations in the spontaneous mutant frequency.

Assays for cytotoxicity and mutagenicity. Solutions of Apur, in medium without horse serum, were freshly prepared. Solutions of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in 5 mM sodium acetate (pH 4.8) were divided into equal samples and stored at −60°C. Exposure to Apur was for 24 h at an initial cell density of 4×10^5 cells per ml. Exposure to MNNG was for 3 h at a cell density of 10^6 cells per ml. Treatments were terminated by centrifugation and suspension of the cells in a drug-free medium. To determine the percent survival, aliquots were diluted and cloned in nonselective semisolid medium (ca. 500 cells per 100-mm-diameter dish) as described previously (11). To determine the frequency of induced mutagenesis, treated cultures and untreated controls were grown for 10 days to allow expression of the mutant phenotype. The mutant frequencies were then determined by selective cloning in semisolid medium (28), and clones in the population resistant to either ouabain, 6-thioguanine, or dexamethasone were quantitated. Ouabain-resistant mutants (OUA^r) were obtained by plating 10^7 cells per 100-mm dish in the presence of 1 mM ouabain; 6-thioguanine-resistant mutants were determined by plating 2×10^6 to 5×10^6 cells per 100-mm dish in 60 μM 6-thioguanine; dexamethasone-resistant mutants were measured by plating 5×10^5 cells per 100-mm dish in 1 μM dexamethasone. Cloning efficiencies were obtained by plating small numbers of cells (200 to 400) in nonselective medium. Colonies were stained and counted after 14 days as described previously (28). The mutant frequencies were obtained by using the following equation: mutant frequency = number of colonies growing in selective medium/(number of cells plated × cloning efficiency in nonselective medium).

The spontaneous mutant frequency, measured in untreated control cultures, was subtracted from the mutant frequency, measured in treated cultures, to give the induced mutant frequency.

Incorporation of ^{3}H-labeled Apur into DNA and RNA. Cells were grown in medium containing 6-^{3}H-labeled Apur (1 to 2.5 mM, 2 to 3 μCi/ml) for 1 to 24 h. Cells were harvested, washed with buffered saline, and suspended in 15 mM $MgCl_2$–25% (vol/vol) ethanol. To determine incorporation into total nucleic acid, we precipitated samples on Whatman GF/C glass filters in chilled 5% trichloracetic acid containing 0.1 M sodium pyrophosphate. The filters were washed with 95% ethanol and dried. The dried filters were digested in 0.3 N NaOH (80°C, 1 h) in scintillation vials, neutralized with acetic acid, and counted in 3a70B counting cocktail (Research Products International). To determine incorporation into DNA, labeled cells were digested in 0.3 N NaOH (1 ml per 2×10^6 cells, 68°C, 1 to 2 h). After being neutralized with HCl, samples were precipitated with trichloroacetic acid as described above. Controls, using [^{3}H]thymidine-labeled cells, showed the DNA to be more than 80% resistant to this treatment, whereas purified ^{32}P-labeled RNA mixed with cells was completely digested.

DNase and RNase digestion of nucleic acid labeled with ^{3}H-labeled Apur. Labeled cells were treated with proteinase K and then extracted with phenol. The protein-free nucleic acid was collected by ethanol precipitation, suspended in 10 mM Tris-hydrochloride (pH 8)–1 mM EDTA, and digested with RNase. Samples were adsorbed on Whatman DE81 paper, washed with 0.1 M pyrophosphate followed by 95% ethanol, and counted. The remaining nucleic acid was further digested with pancreatic DNase I, and samples were counted on DE81 paper, as above.

The ^{3}H-labeled Apur was determined to be greater than 95% pure by thin-layer chromatography on cellulose sheets, using the solvent system *n*-butanol–water–methanol (60:20:20).

Determination of endogenous nucleotide pools. Endogenous nucleotides were extracted and quantitated by high-pressure liquid chromatography as previously described (26, 28).

RESULTS

AraC-6-1 cells are more sensitive to Apur-induced mutagenesis than NSU-1 cells. We assessed the cytotoxic and mutagenic effects of Apur in AraC-6-1 and the parental NSU-1 cells. AraC-6-1 cells were somewhat more sensitive to the cytotoxic effects of Apur (50% effective

concentration, 1.4 mM) than NSU-1 cells (50% effective concentration, 2.0 mM) (Fig. 1).

Figure 2 shows that AraC-6-1 cells were significantly more mutable by Apur at two independent genetic loci. We observed a four- to fivefold increase in the Apur-induced frequency of 6-thioguanine-resistant colonies. (The spontaneous mutant frequency of 6-thioguanine-resistant colonies was $<0.02 \times 10^{-5}$ for NSU-1 and was 0.12×10^{-5} for AraC-6-1.) At the ouabain locus, AraC-6-1 cells were about threefold more mutable by Apur after subtraction of the spontaneous mutant frequency ($<0.01 \times 10^{-5}$ for NSU-1 and 0.05×10^{-5} for AraC-6-1). (AraC-6-1 cells exhibit a high rate of spontaneous mutation [28] as a result of the perturbed dNTP pools. By using frozen samples of a single subclone for all of the Apur mutagenesis experiments, large variations in the value of the spontaneous mutant frequency were avoided.)

Rates of incorporation of ^{3}H-labeled Apur into DNA are the same in AraC-6-1 and NSU-1 cells. ^{3}H-labeled Apur was incorporated into precipitable nucleic acid (DNA + RNA) in a concentration-dependent manner (Fig. 3A). The kinetics of the incorporation (Fig. 3B) and the level of substitution of ^{3}H-labeled Apur in DNA and RNA were the same in AraC-6-1 and NSU-1 cells. Approximately 45% of the total incorporated ^{3}H-labeled Apur was in DNA, as deduced both by resistance to alkali digestion and by sensitivity to appropriate nucleases. The incorporation of ^{3}H-labeled Apur into DNA by both cell lines was approximately 100 pmol per 10^6 cells per 24 h at 2.0 mM ^{3}H-labeled Apur.

Assuming a DNA content of 6 pg per diploid mouse cell (7) and an average molecular weight of 660 per base pair, we calculated that in 24 h (1.4 generation times) approximately 3,200 pmol of adenine is incorporated per 10^6 cells. The relative incorporation of Apur in place of adenine was therefore 3%.

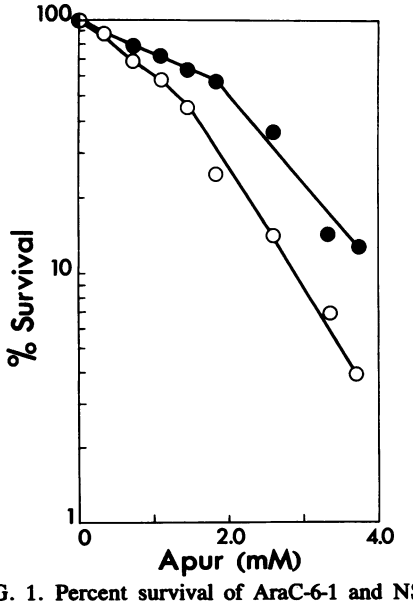

FIG. 1. Percent survival of AraC-6-1 and NSU-1 cells as a function of Apur dose. After being exposed to Apur for 24 h, cells (○, AraC-6-1; ●, NSU-1) were cloned in nonselective semisolid medium (500 cells per 100-mm dish) as described in the text.

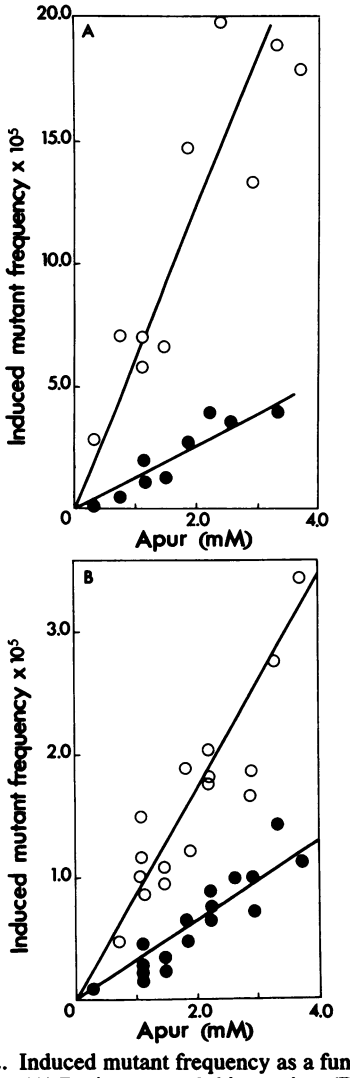

FIG. 2. Induced mutant frequency as a function of Apur dose. (A) Resistance to 6-thioguanine; (B) resistance to ouabain. After being exposed to Apur for 24 h, cells (○, AraC-6-1; ●, NSU-1) were maintained in nonselective medium for 10 days and then cloned in selective, semisolid medium as described in the text. The induced mutant frequency was determined by subtracting the spontaneous mutant frequency (obtained by analyzing untreated cultures) from the total mutant frequency in treated cultures.

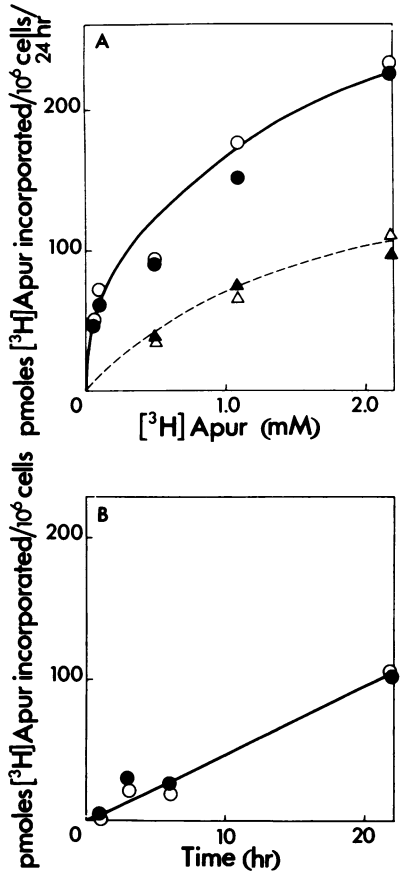

FIG. 3. Incorporation of ^{3}H-labeled Apur into nucleic acid. (A) ^{3}H-labeled Apur incorporation as a function of Apur concentration in the growth medium. (B) Time course of ^{3}H-labeled Apur incorporation. Incorporation of ^{3}H-labeled Apur into total nucleic acid (DNA + RNA) (○, AraC-6-1; ●, NSU-1) or into DNA only (△, AraC-6-1; ▲, NSU-1) was determined as described in the text.

We conclude that the enhanced Apur-induced mutagenesis observed in AraC-6-1 cells is not due to increased incorporation of Apur in that cell line.

Apur does not affect dNTP pools in AraC-6-1 or NSU-1 cells. We examined the effect(s) of Apur on the endogenous nucleotide pools of NSU-1 and AraC-6-1 cells. Under conditions in which Apur was both toxic and mutagenic (1 to 2 mM Apur, 24 h), we observed no significant alteration in the endogenous NTP or dNTP pools (Table 1). Growth arrest, which occurred at the highest dose of Apur, can account for the small drop in the dCTP pools (B. Ullman and S. Eriksson, unpublished data).

AraC-6-1 cells are not more sensitive to mutagenesis by the alkylating agent MNNG. To assess the general ability of AraC-6-1 and NSU-1 cells to repair damaged DNA, we compared their sensitivities to mutagenesis by the alkylating agent MNNG (Table 2). We observed no difference in nitrosoguanidine-induced toxicity or mutagenicity in the two cell lines, suggesting that repair of alkylated DNA was unaffected by the perturbed dCTP and TTP pools in the AraC-6-1 cell line. We conclude that a diminished general ability to repair DNA is not likely to be responsible for the enhanced mutability of AraC-6-1 cells by Apur.

Endogenous TTP and dCTP concentrations modulate the level of Apur-induced mutagenesis in S49 cells. The remaining and most likely factor to account for the enhanced mutability of AraC-6-1 cells by Apur was their perturbed TTP or dCTP (or both) pools. To test this, parallel cultures of NSU-1 and AraC-6-1 cells, growing continuously in the absence or presence of exogenous thymidine (10 or 200 μM), were treated with Apur. Deoxycytidine was added with thymidine to prevent thymidine toxicity by preventing dCTP pool depletion. The levels of the dNTP pools (Table 3) and the induced mutant frequencies (Fig. 4) were determined. The addition of 10 μM thymidine to the growth medium raised the endogenous levels of TTP (from 30 to 42 nmol per 10^9 NSU-1 cells and from 10 to 28 nmol per 10^9 AraC-6-1 cells), reduced the level of dCTP in AraC-6-1 cells (from 223 to 100 nmol per 10^9 cells), and suppressed the difference in mutability of the two cell lines. (The dCTP/TTP ratio was thus reduced from 22.3 to 3.6 in AraC-6-1 cells and from 0.8 to 0.6 in NSU-1 cells.) In the presence of 200 μM thymidine, TTP accumulated to a higher level, approximately 100 nmol per 10^9 cells in both cell lines (thereby reducing the dCTP/TTP ratio to 0.3 in AraC-6-1 cells and to 0.2 in NSU-1 cells). This resulted in low, but equal, levels of Apur-induced mutagenesis in both lines. Figure 5 shows the induced mutant frequency (OUA^r) as a function of endogenous levels of TTP (drawn using data from Table 3 and Fig. 4). A similar curve can be constructed relating the induced mutant frequency to the TTP pool/dCTP pool ratio (not shown). We conclude that the levels of TTP, and possibly also dCTP, modulate the level of mutagenesis induced by Apur.

DISCUSSION

Two mechanisms have been proposed for Apur-induced mutagenesis, one involving base mispairing (10, 14, 23) and the other involving perturbation of endogenous dNTP pools (15). Since a mutant mouse lymphoma cell line (AraC-6-1) containing an abnormally high dCTP pool and a low TTP pool was available, we were able to test both of the proposed mechanisms.

TABLE 1. Effects of Apur on endogenous ribonucleoside and deoxyribonucleoside triphosphate pools[a]

Cell line	Concn of Apur (mM)	nmol per 10^9 cells							
		dCTP	TTP	dATP	dGTP	CTP	UTP	ATP	GTP
NSU-1	0	34	28	24	20	267	600	1,441	293
	1.1	30	34	27	22	282	487	1,438	236
	1.8	27	30	23	13	349	569	1,833	255
AraC-6-1	0	328	18[b]	22	15	291	636	1,497	304
	1.1	288	15	18	12	262	511	1,454	224
	1.8	267	30	22	12	314	565	1,854	306

[a] Intracellular NTP and dNTP concentrations were determined by high-pressure liquid chromatography after 24 h in medium containing Apur.

[b] The observed level of TTP in AraC-6-1 cells was occasionally higher than that previously reported (28). This may be due to the presence of small variable amounts of thymidine in the serum used to make up the culture medium.

One hypothesis, based on in vivo studies with procaryotes (1, 12, 14, 23) and in vitro studies with purified DNA polymerases and synthetic templates (27), proposes that Apur induces transition mutations via formation of Apur:C mispairs during replication. Although both A:T → G:C and G:C → A:T transitions are induced, A:T → G:C transitions are more frequent by a factor of 10 to 20 (6). This hypothesis predicts that the concentrations of the endogenous substrates dCTP and TTP will be an important factor influencing Apur-induced mutagenesis. If this hypothesis is correct, high dCTP or low TTP (or both) would be expected to favor formation of Apur:C mispairs on Apur templates and to enhance the level of mutagenesis. High TTP would favor correct (Apur:T) pairing and suppress mutagenesis.

Our data show that the AraC-6-1 cell line, which contains a low TTP pool and a high dCTP pool, is significantly (three- to fivefold) more mutable by Apur than is its parent cell line, NSU-1. This enhanced mutability was not due to an increase in the level of substitution of Apur in the DNA. Nor was the AraC-6-1 cell line generally impaired in its ability to repair DNA, as evidenced by its normal sensitivity to mutagenesis by the alkylating agent MNNG. We reasoned that, as predicted by one hypothesis (1, 12, 14, 27), the perturbed TTP and dCTP pools were responsible for the increased mutability of AraC-6-1 cells by Apur. This was verified by pharmacologically manipulating the dCTP and TTP pools by adding thymidine to the growth medium. Low concentrations of thymidine (10 μM) increased the low TTP pools (two- to threefold), decreased the high dCTP pools (twofold) in AraC-6-1 cells (thereby decreasing the dCTP/TTP ratio sixfold), and abolished their enhanced mutability by Apur. This result demonstrates that the perturbed dCTP or TTP pool (or both) in AraC-6-1 cells was responsible for its increased sensitivity to Apur-induced mutagenesis. In the presence of high concentrations of thymidine (200 μM), the dCTP and TTP pools in both AraC-6-1 and NSU-1 cells could be

TABLE 2. Cytotoxicity and mutagenicity of MNNG in NSU-1 and AraC-6-1 cells[a]

Cell line	MNNG (μg/ml)	% Survival	6-Thioguanine		Dexamethasone	
			Total mutant frequency ($\times 10^{-5}$)	Induced mutant frequency ($\times 10^{-5}$)	Total mutant frequency ($\times 10^{-5}$)	Induced mutant frequency ($\times 10^{-5}$)
NSU-1	0	100	0.3[b]		119[b]	
	1.7	48	50.5	50.2	256	137
	2.7	18	49.8	49.5	236	117
AraC-6-1	0	100	14.4[b]		229[b]	
	1.7	49	70.4	56.0	405	176
	2.7	13	76.1	61.7	339	110

[a] Cells were treated with MNNG for 3 h. The percent survival and induced mutant frequencies were determined as described in the text.

[b] Continuously cultured cells rather than a recloned population were used in this experiment, accounting for the high spontaneous mutant frequency.

TABLE 3. Manipulation of endogenous levels of dCTP and TTP[a]

Cell line	Addition	nmol/10^9 cells					
		dCTP	TTP	dATP	dGTP	dCTP/TTP	TTP/dCTP
NSU-1	None	23	30	23	17	0.8	1.3
	TdR (10 μM) + dCyt (20 μM)	24	42	23	16	0.6	1.75
	TdR (200 μM) + dCyt (50 μM)	22	113	20	18	0.2	5.14
AraC-6-1	None	223	10	17	10	22.3	0.045
	TdR (10 μM) + dCyt (20 μM)	100	28	20	12	3.6	0.28
	TdR (200 μM) + dCyt (50 μM)	30	91	16	16	0.3	3.03

[a] Cultures were grown continuously in medium supplemented with thymidine (TdR) and deoxycytidine (dCyt) as indicated. Fresh medium was added every 24 h. Endogenous dNTP pools were measured by high-pressure liquid chromatography and were monitored over a 24-h period after the addition of thymidine-containing medium. The values shown represent the average values from three determinations obtained over this period.

perturbed in the reverse direction, producing a high level of TTP relative to dCTP. Although this situation is in itself mutagenic, resulting in elevated spontaneous mutation rates (3), Apur-induced mutagenesis was suppressed. The above observations can be interpreted in two ways. Either the absolute level of the TTP pool or the relative balance of the TTP and dCTP pools determines the level of Apur-induced mutagenesis. Although our data appear to correlate best with the level of TTP, these experiments are insufficient to make a final conclusion. However, these observations support the hypothesis that Apur induces mainly A:T → G:C transitions, the level of mutagenesis being dependent on competition between dCTP and TTP for pairing opposite Apur (incorporated in place of adenine) in the template. Our data do not address the question of possible mismatch repair or editing systems. However, we note that the lack of a linear correlation between the pool perturbation and the altered sensitivity to Apur-induced mutagenesis in AraC-6-1 cells might suggest the involvement of such functions. (A 20- to 30-fold increase in the dCTP/TTP ratio resulted in only a 4- to 5-fold increase in mutability by Apur.)

It has been suggested that in addition to its ambiguous base-pairing properties, Apur is mutagenic by a mechanism involving perturbation

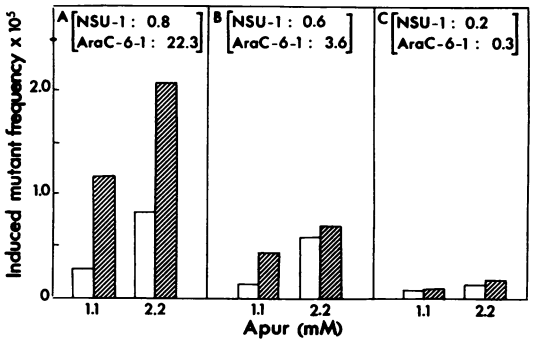

FIG. 4. Induced mutant frequency (OUA^r) after manipulation of endogenous levels of dCTP and TTP. Parallel cultures were maintained, for the duration of the experiment, in medium containing (A) no addition, (B) 10 μM thymidine + 20 μM deoxycytidine, or (C) 200 μM thymidine + 50 μM deoxycytidine. The frequency of cells resistant to ouabain (or 6-thioguanine, data not shown) was determined after exposure to Apur for 24 h (▨, AraC-6-1; □, NSU-1). The dCTP/TTP ratios calculated from the data in Table 3 are given in parentheses.

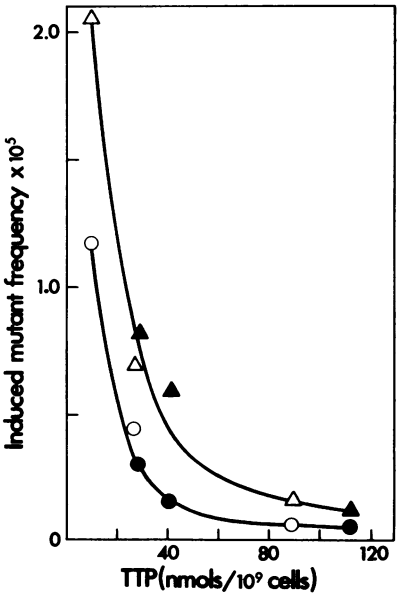

FIG. 5. Induced mutant frequency (OUA^r) as a function of endogenous levels of TTP. Data from Table 3 and Fig. 4 were replotted. Symbols: (○) AraC-6-1 and (●) NSU-1 after being exposed to 1.1 mM Apur; (△) AraC-6-1 and (▲) NSU-1 after being exposed to 2.2 mM Apur.

of the endogenous deoxynucleotide pools (15). We observed no significant changes in the deoxynucleotide pools after exposure of cells to mutagenic concentrations of Apur. Apur differs in this respect from another well-characterized base-analog mutagen, bromodeoxyuridine (15).

Meuth (19) recently reported that the balance of dCTP and TTP in CHO cells affects their sensitivities to DNA-alkylating agents. Increasing the TTP/dCTP ratio by adding thymidine to the growth medium promoted mutagenesis by ethyl methane sulfonate, suggesting that mutagenesis may involve formation of O^6-methylguanine:T mispairs. However, the ethyl methane sulfonate-mediated induction of 6-thioguanine resistance in the Thy^- mutant described by Meuth (which resembles AraC-6-1 in having an abnormally high dCTP/TTP ratio) was elevated about 10-fold. This observation is not consistent with a model implicating exclusively O^6-methylguanine in ethyl methane sulfonate mutagenesis, possibly suggesting that other mechanisms may also be involved. The AraC-6-1 mouse lymphoma cell line apparently differs from Thy^- in its response to alkylating agents, the former showing no alteration in sensitivity as a result of the perturbed dCTP/TTP ratio. These studies indicate the general usefulness of such mutants to probe the mechanism of action of various mutagens.

A current picture that is emerging suggests that balanced dNTP pools are necessary to maintain accurate replication of cellular DNA and hence low spontaneous mutation rates. Unbalanced dNTP pools lead to decreased replicative fidelity in cell-free procaryotic systems (8, 13, 17, 18, 29) and mutator phenotypes in cultured mammalian cells (21, 28). It now appears that the dNTP pools can, in some cases, also modulate the sensitivity of cells to chemically induced mutagenesis.

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