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SYNERGISTIC AND ANTAGONISTIC INTERACTIONS OF METHOTREXATE AND 1-β-D-ARABINOFURANOSYLCYTOSINE IN HEPATOMA CELLS

THE MODULATING EFFECT OF PURINES*

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Abstract—A 4-hr pretreatment with methotrexate antagonized the cytotoxic effect of subsequent arabinosylcytosine treatment in rat hepatoma cells of lines N_1S_1 and 3924A, but in the hepatoma line 8999R and the fibroblast line BF5, MTX pretreatment was synergistic with the arabinosylcytosine treatment. Measurement of cellular deoxyribonucleoside triphosphate concentrations showed that in those lines in which antagonism was found the dCTP increased, whereas in the lines where the drugs were synergistic the dCTP pool was decreased. Conversely, dATP levels were high when the drugs were synergistic and low when antagonism was obtained. Although methotrexate pretreatment antagonized arabinosylcytosine in N_1S_1 and 3924A cells, pretreatment of these cells with the combination of methotrexate plus a purine (either hypoxanthine or 2′-deoxyadenosine) resulted in synergism with arabinosylcytosine. Deoxynucleotide pool measurements showed that methotrexate in combination with either hypoxanthine or 2′-deoxyadenosine increased dATP and decreased dCTP in the N_1S_1 and 3924A hepatoma cells. In N_1S_1 cells, pretreatment with 2′-deoxyadenosine alone for 4 hr was synergistic with arabinosylcytosine. It was concluded that elevated dATP pools enhanced arabinosylcytosine cytotoxicity by depleting the dCTP pool, through feedback inhibition of ribonucleotide reductase, thus causing greater inhibition of DNA biosynthesis and greater incorporation of AraCTP into nucleic acid. Methotrexate was synergistic in those cell lines where dATP accumulated and dCTP was decreased, but when methotrexate had a potent antipurine effect dCTP pools increased and arabinosylcytosine was antagonized. The synergistic interaction was more marked at cytocidal drug concentrations than it was at growth-inhibitory doses.

Methotrexate (MTX)‡ and arabinosylcytosine (Ara-C) are antineoplastic drugs of clinical importance, and the two agents are sometimes used in combination [1, 2]. Experimental studies of the interaction of MTX and Ara-C have given conflicting results. An early *in vivo* study with mouse leukemia L1210, using simultaneous administration of the two drugs, indicated that the effects were infra-additive; at relatively high doses the prolongation of survival given by the combination was greater than that caused by either agent used separately, but at lower doses the effect of the combination was no better than that of Ara-C alone [3]. A study of the time–sequence dependence of the combined effect in the TLX5 mouse lymphoma showed that when MTX was administered after Ara-C the results were additive, but when MTX preceded Ara-C potent antagonism was obtained [4]. Avery and Roberts [5], using simultaneous administration, obtained a synergistic effect against L1210 *in vivo*, and the degree of synergism was greater at the highest drug doses. Cadman and Eiferman [6], giving MTX 6 hr before Ara-C, obtained results that were better than those given by either drug singly, again in L1210.

Results of *in vitro* studies of MTX Plus Ara-C have been equally varied. Chu and Fischer [7] found that MTX pretreatment increased the cytotoxicity of Ara-C to L5178Y mouse lymphoma cells *in vitro*, and Hoovis and Chu [8] reported that MTX treatment increased the accumulation of Ara-C metabolites in the L5178Y cells. Roberts *et al.* [9], working with L1210 leukemia cells *in vitro*, found increased cellular AraCTP pools following MTX treatment, and correlated this finding with increased activity of deoxycytidine kinase. Cadman and Eiferman [6] claimed that MTX caused increased uptake of Ara-C in a number of cell lines. Edelstein *et al.* [10], using a mouse spleen colony assay, reported that the MTX plus Ara-C combination had enhanced therapeutic selectivity because combined effects on the colony forming ability of L1210 cells were greater than additive whilst effects on mouse bone marrow cells were less than additive. In contrast to these results, Grindey and Nichol [11], working with sublethal drug concentrations, found that amounts of

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‡Abbreviations used in the test: MTX, methotrexate (amethopterin); Ara-C, 1-β-D-arabinofuranosylcytosine; AraCTP, 5′-triphosphate of Ara-C; dNTP, deoxyribonucleoside triphosphate; and AdR, 2′-deoxyadenosine.

MTX and Ara-C required to give a combined growth inhibition of 50 per cent were infra-additive. This finding was confirmed in the L5178Y lymphoma by Tattersall and Harrap [12] who suggested that the lack of additivity of the growth-inhibitory effects was the consequence of dCTP accumulation caused by the MTX; this would competitively inhibit the effect of AraCTP on DNA polymerase and other possible target sites.

The present report describes an attempt to devise and test a unifying hypothesis that would explain as many as possible of these apparently conflicting observations. Rat hepatoma cells were chosen as the primary test system because a series of lines are available that cover a range of growth rates, and because the regulation of their nucleotide metabolism has been extensively characterized.

MATERIALS AND METHODS

Chemicals. MTX, Ara-C, nucleosides and nucleotides were purchased from the Sigma Chemical Co., St. Louis, MO. [^{3}H]Ara-C was obtained from Amersham/Searle, Arlington Heights, IL. High pressure liquid chromatography columns were from Whatman Inc., Fair Lawn, NJ. Tissue culture supplies were from the Grand Island Biological Co., Grand Island, NY.

Cell culture. Cell lines were maintained in McCoy's medium 5A supplemented with 5% dialyzed horse serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Novikoff hepatoma cells of line N_1S_1-67 were grown in stationary suspension culture; their mean log-phase doubling time under these conditions was 11 hr. For cloning studies a monolayer variant of the N_1S_1-67 line was used. Morris hepatoma cells of line 3924A were grown in monolayer culture, with a mean log-phase doubling time of 15 hr. Morris hepatoma 8999R grew as a monolayer with a doubling time of 35 hr. BF5 (rat skin fibroblast) cells grew in monolayer culture with a mean doubling time of 28 hr. The origin and properties of these various cell culture lines were summarized previously [13]. For growth inhibition experiments 10 ml cultures were initiated in 25 cm^2 flasks (Falcon Plastics, Oxnard, CA) at a cell density of 3×10^4/ml. For cloning studies, 500 cells in 10 ml of growth medium were seeded in a culture flask and maintained in a 95% air + 5% CO_2 atmosphere in a humid incubator. After 1 week the growth medium was decanted, and the cells were rinsed in isotonic phosphate-buffered saline and stained for 5 min in saturated crystal violet solution in isotonic saline. Colonies were counted visually. Cloning efficiency of untreated cells under these conditions was 70 per cent for N_1S_1, 60 per cent for 3924A, 40 per cent for 8999R and 35 per cent for BF5. Drug interactions were classified as follows: *Summation*, as defined by Webb [14], indicates that fractional inhibition given by a combination of two drugs was the product of the fractional inhibitions given by the two agents used separately. *Synergism* indicates a combined effect greater than summation. *Antagonism* implies a combined effect less than that given by either agent alone, and *infra-additivity* is the situation where the combination was less than summative, but more effective than either agent used separately.

Deoxyribonucleotide assays. Deoxyribonucleoside triphosphates were assayed in 60% methanol extracts prepared by the method of Tyrsted [15] as modified by Harrap and Paine [16]. dGTP and dCTP were measured by the method of Solter and Handschumacher [17] using native calf thymus DNA as template-primer. dTTP and dATP were assayed according to Lindberg and Skoog [18], using poly(dAdT) as template-primer.

Analysis of Ara-C metabolites. Cells were incubated for the appropriate time in medium containing tritiated Ara-C. About 2×10^7 cells were harvested by centrifugation at 1500 *g* for 5 min, and cell pellets were extracted in 1 ml of ice-cold 0.7 N perchloric acid. Extracts were centrifuged at 20,000 *g* for 20 min to remove precipitated protein. Perchlorate was removed from the supernatant fraction by addition of 75 mg potassium bicarbonate, followed by centrifugation at 1500 *g* for 5 min. The extracts were then brought to pH 2.6 by addition of 25 μl of 4 N HCl. Two hundred μl aliquots were analyzed by high pressure liquid chromatography on a Whatman Partisil PSX 10/25 SAX anion exchange column, 25 × 0.46 cm, using buffer conditions described by Lui *et al.* [19]. On this column the Ara-C nucleotides co-eluted with their cytidine analogues. The column eluent was conducted to a fraction collector loaded with miniature scintillation vials containing a high water-capacity liquid scintillation mixture, and 1-min fractions were collected and counted.

Deoxycytidine kinase assays. Approximately 5×10^7 cells were harvested by centrifugation at 1500 *g* for 5 min, and 1.5 ml of extraction medium consisting of 0.05 M Tris-HCl, pH 8.0, containing 0.5 mM dithioerythritol was added to the cell pellet. Cells were lysed by three cycles of freezing in liquid nitrogen followed by thawing at 37°. Lysates were centrifuged at 105,000 *g* for 30 min, and assays were performed on the supernatant fraction. The reaction mixture contained, in a final volume of 0.15 ml, 20 mM Tris–HCl (pH 8.0), 50 μl of the enzyme preparation, 0.2 mM dithioerythritol, 1 mM KCl, 0.7 mM $MgCl_2$, 7 mM neutralized ATP, 1 mM phosphoenolpyruvate, 10 I.U. pyruvate kinase, and 0.4 mCi/mmole [^{3}H]deoxycytidine. This mixture, in a 400 μl microcentrifuge tube, was incubated for 20 min at 37°. The reaction was stopped by boiling for 5 min, and precipitated protein was sedimented by a 30-sec spin in the microcentrifuge. Aliquots of the supernatant fraction (50 μl) were applied to 2 × 2 cm polyethyleneimine cellulose discs (Polygram Cel 300 PEI). Unreacted deoxycytidine was removed by three successive 5-min washes in 150 ml of 1 mM ammonium formate, followed by a 5-min wash in 150 ml of water, and a 5-min wash in 95% ethanol. After drying, the discs were placed in scintillation vials containing 10 ml of scintillation fluid (OCS, Amersham) and counted. Reaction blanks using boiled enzyme were counted. Counting efficiency was determined by counting unwashed discs.

RESULTS

The results of a preliminary examination of the

Table 1. Inhibition of growth of hepatoma cells by Ara-C and MTX*

Treatment	Number of cell doublings in 72 hr			
	3924A		8999R	
None	4.79	(100)†	2.21	(100)
Ara-C (800 nM)	2.68	(56)	0.99	(45)
MTX (20 nM)	2.11	(44)	1.30	(59)
Ara-C (800 nM), then MTX (20 nM), 4 hr later	1.29	(27)	0.62	(28)
Ara-C (800 nM) and MTX (20 nM), simultaneously	1.68	(35)	0.57	(26)
MTX (20 nM), then Ara-C (800 nM), 4 hr later	2.20	(46)	0.40	(18)

* Cells were grown in continuous presence of the drugs for 72 hr and then counted on a Coulter counter. If the drug effects showed simple summation, the predicted growth in the presence of the combination would have been $0.56 \times 0.44 \times 100 = 25$ per cent control for 3924A, and $0.45 \times 0.59 \times 100 = 27$ per cent control for 8999R.

† Numbers in parentheses indicate values as percentages of untreated control. Values are means of triplicate cultures.

combined effects of MTX and Ara-C on two hepatoma lines are shown in Table 1. This experiment measured growth over a 72-hr period in continuous presence of the drugs, using drug concentrations that, when used alone, gave about 50 per cent growth inhibition. When MTX was added to the cultures 4 hr after Ara-C, the drug effects gave summation in both cell lines. If the two agents were added to the cultures simultaneously, summation was still obtained in the hepatoma 8999R, but in the more rapidly growing 3924A cells the combination was infra-additive. A pronounced difference in the behaviour of the two cell lines became apparent when administration of MTX to the cultures preceded Ara-C by 4 hr. Under these conditions the combined effect in 3924A cells was no greater than that given by MTX alone, but synergism was obtained in 8999R cells (Table 1).

Since the use of MTX 4 hr before Ara-C gave wide differences between the two cell lines, this regimen was chosen for further study. Table 2 shows the effect of this combination on four rat cell lines. In these experiments we examined cell killing, rather than growth inhibition, and drug concentrations lethal to approximately 50 per cent of cells (LD_{50}) were chosen. After the 4-hr MTX pretreatment (where applicable), Ara-C was added to the cultures which were incubated for a further 6 hr. Then about 500 cells were transferred to flasks containing 10 ml of fresh, drug-free medium for determination of cloning efficiency. In the rapidly growing N_1S_1 and 3924A hepatoma cell lines, the MTX + Ara-C combination gave antagonistic results, whereas in the more slowly growing BF5 fibroblasts and 8999R hepatoma cells the combination was synergistic.

It seemed possible that the influence of MTX on cellular response to Ara-C may have been mediated through the pool of dCTP, so the effect of a 4-hr MTX treatment (0.5 μM) on pools of the four deoxyribonucleoside triphosphates was examined in the four cell lines. Results are shown in Table 3. In the two cell lines in which MTX and Ara-C were antagonistic (N_1S_1 and 3924A), the dCTP pool increased in response to MTX, whereas in the two lines where the agents were synergistic (BF5 and 8999R) MTX treatment caused a decrease in the dCTP pool. The dTTP pool was decreased by MTX in all four cell lines. In the two rapidly growing cell lines, MTX also had an antipurine effect, causing marked decreases in dGTP and a lesser drop in the dATP pool. In the two more slowly growing cell lines, MTX did not have an antipurine effect, the dGTP pools were unchanged or increased, and dATP was elevated in both cases (Table 3).

Table 2. Effects of Ara-C and MTX on colony-forming ability of rat cell lines*

Treatment	Cloning efficiency (% of untreated control)			
	N_1S_1	3924A	BF5	8999R
Ara-C (15 μM), 6 hr	43	57	54	42
MTX (0.5 μM), 4 hr	40	48	52	55
MTX (0.5 μM), 4 hr, then Ara-C (15 μM), 6 hr	48	59	19	13
Predicted values for summation	17	27	28	23

* Following treatment, 500 cells were transferred to drug-free medium, and colonies were counted after 1 week, as described in Material and Methods. The final line of the table shows predicted cloning efficiency assuming Ara-C and MTX effects are summative, i.e. efficiency (Ara-C) × efficiency (MTX) × 100.

Table 3. Deoxyribonucleoside triphosphate pools in MTX-treated cells

Cell line	Treatment	Deoxyribonucleoside triphosphate content (nmoles/10^9 cells)			
		dTTP	dCTP	dATP	dGTP
N_1S_1	None	47	12	17	9
	MTX (0.5 μM), 4 hr	15 (32)*	25 (208)	12 (71)	3 (33)
3924A	None	40	18	14	11
	MTX (0.5 μM), 4 hr	11 (28)	40 (222)	11 (79)	6 (55)
BF5	None	28	17	12	5
	MTX (0.5 μM), 4 hr	10 (36)	11 (65)	21 (175)	5 (100)
8999R	None	31	12	15	6
	MTX (0.5 μM), 4 hr	17 (55)	4 (33)	36 (240)	11 (183)

* Numbers in parentheses indicate the MTX-treated values as percentages of the appropriate control.

Table 4. Effects of Ara-C and MTX on colony-forming ability of hepatoma cells in the presence of purines*

Treatment	Number of colonies (% control)	
	N_1S_1	3924A
MTX (0.5 μM)	38	46
Ara-C (15 μM)	41	55
MTX (0.5 μM) + Hx (100 μM)	43	57
MTX (0.5 μM) + AdR (100 μM)	37	44
MTX (0.5 μM), then Ara-C (15 μM), 4 hr later	43	56
MTX (0.5 μM) + Hx (100 μM), then Ara-C (15 μM), 4 hr later	13	20
MTX (0.5 μM) + AdR (100 μM), then Ara-C (15 μM), 4 hr later	10	16

* Following treatment, 500 cells were transferred to drug-free medium, and colonies were counted after 1 week, as described in Materials and Methods. Hx = hypoxanthine.

The antagonism of MTX and Ara-C thus correlated with increased cellular dCTP and with the antipurine effect of MTX. This suggested the possibility that if the antipurine effect of MTX could be abolished, by coadministration of a purine base or nucleoside, then the antagonism between MTX and Ara-C in the rapidly growing cell lines might be decreased or abolished. Results of an experiment designed to test this hypothesis are summarized in Table 4. In this study, N_1S_1 and 3924A hepatoma cells were preincubated for 4 hr with either MTX (0.5 μM) plus hypoxanthine (100 μM) or MTX (0.5 μM) plus 2′-deoxyadenosine (100 μM). Then Ara-C (15 μM) was added to the cultures and, after a further 6 hr, aliquots of 500 cells were transferred to drug-free medium for the colony-forming assay. Under these conditions the pretreatment with MTX alone did not increase the cytotoxic effectiveness of Ara-C, but pretreatment with MTX plus either hypoxanthine or 2′-deoxyadenosine was strongly synergistic. A possible interpretation for these data was suggested by the measurements of deoxynucleoside triphosphate pools in N_1S_1 cells treated with the MTX + purine combination (Table 5). MTX alone,

Table 5. Deoxyribonucleoside triphosphate pools in N_1S_1 cells treated with MTX and purines*

Treatment	Deoxyribonucleoside triphosphate content (nmoles/10^9 cells)			
	dTTP	dCTP	dATP	dCTP
None	52	16	18	10
MTX (0.5 μM), 4 hr	24 (46)†	24 (150)	14 (78)	5 (50)
MTX (0.5 μM) + Hx (100 μM), 4 hr	19 (37)	11 (69)	29 (161)	19 (190)
MTX (0.5 μM) + AdR (100 μM), 4 hr	15 (29)	6 (38)	42 (233)	16 (160)

* Values are means of triplicate cultures.

† Numbers in parentheses indicate the treated values as percentages of the untreated control.

Table 6. Arabinosylcytosine 5'-triphosphate pools in MTX-treated hepatoma cells*

Pretreatment	AraCTP (nmoles/10^9 cells)	
	N_1S_1 cells	8999R cells
None	0.117 ± 0.014	0.071 ± 0.012
MTX (20 nM), 4 hr	0.148 ± 0.035 (126)†	0.090 ± 0.003‡ (127)
MTX (0.5 μM), 4 hr	0.163 ± 0.031‡ (139)	0.104 ± 0.009‡ (146)

* Cultures were preincubated with the appropriate concentration of MTX, then [^{3}H]arabinosylcytosine (0.5 Ci/mmole) was added to the medium at a final concentration of 0.1 μM, and cultures were incubated for a further 4 hr. Cells were extracted and Ara-CTP was assayed as described in Materials and Methods.

† Values in parentheses indicate treated values as percent of untreated control.

‡ Significantly greater than control ($P < 0.05$ in the two-tail Student's t-test).

Table 7. Activity of deoxycytidine kinase in rat hepatoma and fibroblast cells following MTX treatment

Treatment	Deoxycytidine kinase activity [nmoles·hr^{-1}·(mg protein)$^{-1}$]	
	N_1S_1	BF5
Control	16.8 ± 2.6	12.0 ± 0.7
MTX (20 nM), 4 hr	19.2 ± 2.0 (114)†	13.2 ± 2.1 (110)
MTX (0.5 μM), 4 hr	9.6 ± 0.1 (57)	10.3 ± 1.0 (86)

* Assay procedures for enzyme activity and total cytosol protein are described in Materials and Methods.

† Numbers in parentheses indicate values as percentages of the untreated controls.

as observed in the previous experiment reported in Table 3, gave marked decreased in dTTP and dGTP, a lesser decrease in dATP, and an increase in the dCTP pool. The combination of MTX with a purine, either hypoxanthine or deoxyadenosine, increased the depression of the dTTP pool, and abolished the antipurine effect of MTX. Moreover, whereas MTX alone gave increased dCTP after 4 hr, the MTX + purine combination resulted in a decreased dCTP pool (Table 5). Because it seemed likely that the response of cells to Ara-C might depend on the ratio of the competing metabolite and antimetabolite, dCTP and AraCTP, we examined the effect of a 4-hr pretreatment with two MTX concentrations (growth-inhibitory and cytotoxic) on subsequent phosphorylation of Ara-C in two hepatoma cell lines, the N_1S_1 (in which MTX pretreatment was antagonistic with Ara-C) and the 8999R (in which the

Table 8. Cytotoxic effects of combinations of MTX, Ara-C, and 2'-deoxyadenosine on N_1S_1 hepatoma cells*

First agent	Second agent	Third agent	Number of colonies (% control)
AdR			81 ± 2
MTX			43 ± 4
Ara-C			46 ± 4
AdR	MTX		39 ± 2
MTX	AdR		48 ± 5
AdR	Ara-C		25 ± 3
Ara-C	AdR		36 ± 1
MTX	Ara-C		52 ± 7
Ara-C	MTX		24 ± 3
AdR	MTX	Ara-C	14 ± 1
AdR	Ara-C	MTX	8 ± 2
MTX	AdR	Ara-C	16 ± 3
MTX	Ara-C	AdR	39 ± 7
Ara-C	MTX	AdR	22 ± 3
Ara-C	AdR	MTX	19 ± 4

* Values are means for triplicate cultures, expressed as percentages of quadruplicate control counts, followed by S.E.M. The interval between administration of successive drugs was 4 hr. Drug concentrations were: MTX, 0.5 μM; Ara-C, 15 μM; and AdR, 100 μM.

combination was synergistic). In both cell lines, MTX pretreatment at both concentrations caused small increases in AraCTP pools (Table 6).

Table 7 shows effects of 4-hr MTX pretreatment (at inhibitory and lethal doses) on activity of deoxycytidine kinase in two of the cell lines. No significant increases in activity were found in any of these experiments.

The results shown in Tables 4 and 5 demonstrated that addition of a purine during the MTX pretreatment could change the MTX–Ara-C interaction from antagonism to synergism, perhaps because abolition of the MTX antipurine effect led to decreased dCTP pools. Additional support for this concept is provided by the data of Table 8, in which a 4-hr pretreatment with 2′-deoxyadenosine (without MTX) was shown to increase the cytotoxic effect of Ara-C in N_1S_1 cells. All possible combinations of the three agents, Ara-C, MTX and 2′-deoxyadenosine, were tested, separated by 4-hr intervals, and the most effective ternary combination was found to be 2′-deoxyadenosine, followed by Ara-C, followed by MTX (Table 8).

DISCUSSION

AraCTP inhibits DNA polymerase by competition with the incorporation of dCTP [20–23] and it is also incorporated into DNA and RNA [7, 23, 24]. Since the first two effects are opposed by dCTP, the ratio of dCTP to AraCTP must be a determinant of Ara-C cytotoxicity. Previous studies found infra-additive effects when MTX was combined with Ara-C, suggesting that MTX reduced the efficacy of Ara-C by causing increased cellular dCTP, which was competitive with AraCTP [11, 12]. Our present results support this explanation: in two cell lines where MTX elevated the dCTP pool, MTX and Ara-C gave infra-additive or antagonistic effects on growth inhibition and on cell killing. However, in two cell lines where MTX and Ara-C gave summation or synergism, MTX treatment resulted in decreased dCTP pools (Tables 1–3). It remains to explain why MTX should increase the dCTP pool in some cells and decrease it in others. Evidence from studies with the ribonucleotide reductase of rat hepatoma cells and calf thymus indicates that production of deoxycytidylate nucleotides is regulated by the pools of dATP and dTTP [25, 26]. The effect of MTX on dCTP is thus probably a secondary consequence of the effects on dTTP and dATP. MTX consistently decreases dTTP in mammalian cells [27–33], which would increase dCTP production by ribonucleotide reductase, but would also increase activity of dCMP deaminase [29]. The net effect on dCTP pools could be an increase or decrease, depending upon the detailed kinetic properties of CDP reductase and dCMP deaminase in various cell lines. dATP is a more potent negative effector of ribonucleotide reductase than dTTP [25, 26]. The effect of MTX on dATP pools varies, depending on how extensive the antipurine effect of MTX is in the cell line under study. In L5178Y lymphoma cells MTX has a potent antipurine effect [28, 34] and the dATP pool does not increase after MTX treatment [27, 28], though in other cell lines where the antipurine effect of MTX is less marked the block in DNA polymerase activity resulting from lack of dTTP causes the dATP pool to rise [28, 30]. The antipurine effect of MTX is most marked in cells of rapid growth rate [34, 35], and accumulation of dATP following MTX is greater in cells of slower growth rate. Our results (Table 3) showed decreased or unchanged dATP in the rapidly growing N_1S_1 and 3924A lines and increased dATP in the slowly growing 8999R and BF5 cells. In these lines the change in dCTP pools correlated inversely with the dATP pool. Similarly, Fridland [29] and Tattersall *et al.* [30], working with lymphoblasts, showed increased dATP and decreased dCTP after MTX treatment. The results of Hoovis and Chu [8] appeared to contradict this generalization, since they found that MTX was synergistic with Ara-C in L5178Y cells, in which MTX has a strong antipurine effect. In their study, however, the growth medium contained hypoxanthine, which neutralized the antipurine effect of MTX. Consequently, we examined the effect of added purines on the MTX–Ara-C interaction in hepatoma cells. In growth-inhibition experiments with N_1S_1 cells (results not shown), addition of deoxyadenosine or hypoxanthine did not alter the MTX–Ara-C interaction, which remained infra-additive, as found by Grindey and Nichol [11] with L1210 cells. At cytotoxic drug concentrations, however, addition of a purine changed the nature of the interaction in N_1S_1 and 3924A cells from infra-additive to synergistic (Table 4), and the dNTP pool measurements (Table 5) confirmed that, in the presence of deoxyadenosine, treatment with a cytotoxic dose of MTX resulted in lowered dCTP.

In the present work, the emphasis has been on studying the effect of MTX on the dCTP concentration, but it is clear that, in the competitive relationship between cellular pools of dCTP and AraCTP, the pool size of AraCTP is at least as important in determining the nature of MTX–Ara-C interactions. As previously described in other cell lines [6, 8, 9] we found increased AraCTP accumulation after MTX pretreatment (Table 6), but unlike the situation in L1210 cells [9] no marked increases were seen in deoxycytidine kinase activity (Table 7). The increased AraCTP in the BF5 cells was possibly the result of release of deoxycytidine kinase from feedback inhibition by dCTP.

Earlier studies showed that the interaction between MTX and Ara-C became more synergistic at higher drug concentrations [3, 5, 10] and our results support this observation (Tables 1 and 2). A possible biochemical basis for this is suggested by the results of Kinahan *et al.* [33] who showed that growth of L1210 cells could be inhibited up to 70 per cent without a fall in dCTP, but that further inhibition by MTX led to decreased dCTP. Similarly, Skoog *et al.* [31] showed that 2-OS osteosarcoma cells, after 4 days in 3 nM MTX, had elevated dCTP, but after 4 days in 100 nM MTX has dCTP below control. Metabolite pools represent a balance between production and utilization. It is possible that sometimes MTX may have an antipurine effect, decreasing the dATP pool, but if high concentrations of Ara-C are then added, DNA polymerase may be inhibited to an extent such that even though the rate of dATP biosynthesis is below normal, it will still be

greater than the rate of dATP incorporation into DNA, so that the dATP pool increases with resulting inhibition of CDP reductase and decreased dCTP.

The interaction of MTX and Ara-C is thus a multifactorial problem. The two agents have opposing effects upon the dTTP pool, which influences both dCTP production and dCMP deamination, and MTX has different effects upon the dATP pool in different cell lines. The present report has emphasized the role of purines in modulating the effect of MTX on the dCTP pool, and it has shown that pretreatment with MTX plus a purine gave synergism with Ara-C even in cell lines where MTX alone antagonized Ara-C. In a clinical situation, however, the anti-purine effect of MTX may be desirable. An optimal combination may then be that in Table 8, experiment 11, i.e. deoxyadenosine, followed by Ara-C, followed by MTX. This protocol is now being evaluated in detail.

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