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EFFECTS OF METHOTREXATE ON PURINE AND PYRIMIDINE METABOLISM AND CELL-KINETIC PARAMETERS IN HUMAN MALIGNANT LYMPHOBLASTS OF DIFFERENT LINEAGES*

JOS P. M. BÖKKERINK,† RONNEY A. DE ABREU, MARINKA A. H. BAKKER, TILLY W. HULSCHER, JOHN M. VAN BAAL, EGBERT D. A. M. SCHRETLEN and CHRIS H. M. M. DE BRUIJN‡

Centre for Pediatric Oncology S.E. Netherlands, Department of Pediatrics, St Radboud Hospital, University of Nijmegen, 6500 HB Nijmegen, The Netherlands

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Abstract—MOLT-4 (T-), RAJI (B-), and KM-3 (non-B-non-T-, common ALL) malignant lymphoblasts demonstrated significant differences in their activities of purine *de novo* synthesis (PDNS) and purine salvage pathway and in their cell-kinetic parameters. Incubations with concentrations of methotrexate (0.02 and 0.2 μM), which can be maintained during many hours in the oral maintenance therapy of acute lymphoblastic leukemia, indicated large differences between the three cell lines with respect to the inhibition of PDNS, depending on the concentration of methotrexate (MTX) and on the activities of the two pathways. These dose- and cell line-dependent differences corresponded to the perturbations of cell-kinetics and purine and pyrimidine (deoxy)ribonucleotide pools in the three cell lines.

Exposure of MOLT-4 cells to 0.02 μM MTX resulted in an incomplete inhibition of DNA synthesis in early S phase, as shown by DNA-flow cytometry and increase of dCTP levels, which recovered spontaneously after 48 hr. Almost no impairment of RNA synthesis occurred (unbalanced growth). In RAJI cells, exposed to 0.02 μM MTX, DNA synthesis was delayed in the S phase, not arrested, and RNA synthesis was not impaired, also indicating an unbalanced growth pattern, which, however, did not recover in time. KM-3 cells were arrested in G1 phase and subsequently in early S phase after incubation with 0.02 μM MTX, and perturbations of ribonucleotides indicated a complete inhibition of RNA synthesis, resulting in a balanced growth pattern. Cytotoxicity was more pronounced in KM-3 cells. The reliability of the soft agar colony forming assay after low dose MTX treatment is discussed.

Exposure of MOLT-4 and KM-3 cells to 0.2 μM MTX resulted in a complete inhibition of DNA synthesis, with cessation of cell progression through all parts of the cell cycle and arrest in G1 phase. RAJI cells showed an increasing accumulation of cells in G1 phase without complete cessation of cell cycle progression. Perturbations of ribonucleotide pools suggested an inhibition of RNA synthesis in all cell lines, indicating a balanced growth pattern in KM-3 cells and MOLT-4 cells. Increased levels of ribonucleotides after prolonged exposure to 0.2 μM MTX were due to reutilization of purine and pyrimidine precursors from nucleic acid breakdown consuming PRPP, whereas consumption of ribonucleotides for RNA synthesis was absent. The perturbations of deoxyribonucleotide pools after exposure to 0.02 and 0.2 μM MTX were in agreement with the changes in DNA-flow cytometry. Increased levels of dCTP after exposure to 0.02 μM MTX correlated with accumulation of cells in early S phase. A conspicuous phenomenon was the absence of decrease in dGTP pools in RAJI cells, in which cytotoxicity was low. Cytotoxicity was correlated with the degree of PDNS inhibition and a severe decrease of both dGTP and dTTP pools.

The differences between the T-, B-, and non-B-non-T-, common ALL cell lines with respect to the parameters studied may explain the different responses to treatment of patients with corresponding lymphoid leukemias and lymphomas and suggest possibilities for different treatment strategies.

Methotrexate (MTX) is a well established chemotherapeutic agent in the treatment of all immunological subclasses of acute lymphoblastic leukemia (ALL) and non-Hodgkin's malignant lymphoma in childhood [1–3]. It is used in high dose intravenous chemotherapy and prophylactic central nervous system treatment [4]. The combination of MTX and 6-mercaptopurine (6MP) is widely used in the oral maintenance therapy of non-B ALL and non-Hodgkin's lymphoma [1–3].

In a previous study [5], we demonstrated a synergistic action of the combination of MTX and 6MP in the three human malignant lymphoblastic cell lines MOLT-4 (T-lineage), RAJI (B-lineage), and KM-3 (non-B-non-T-, common ALL cells). The sequence-, dose-, and time-dependent synergism was based on the inhibition of purine *de novo* synthesis (PDNS) after treatment with MTX, resulting in increased availability of 5-phosphoribosyl-1-pyrophosphate (PRPP). The increased availability of PRPP could be used for an enhanced conversion of 6MP by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). We demonstrated differences with

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† To whom requests for reprints should be addressed.

‡ Present address: Department of Biomedical and Health Care Technology, University of Technology, Eindhoven, The Netherlands.

respect to this synergism between MOLT-4 cells and RAJI and KM-3 cells, due to differences in the activities of the PDNS and the purine salvage pathway in untreated cells. In addition, this selective synergism in malignant lymphoblasts will not be observed in normal bone marrow cells and peripheral blood lymphocytes with a low activity of PDNS [6–10].

Many studies [11–26] described the effects of MTX on cytotoxicity, cell-kinetic parameters and purine and pyrimidine deoxyribonucleotide pools in bone marrow, stimulated peripheral blood lymphocytes and various malignant cell lines. However, according to our knowledge, correlations between these effects of MTX and differences in inhibition of purine *de novo* synthesis and ribonucleotide pools between various malignant lymphoblasts are scarcely available. This especially holds for concentrations of MTX which can be maintained *in vivo* for many hours during oral maintenance therapy of ALL in children [27].

In the present study we have compared the sequential time-dependent effects of 0.02 μM and 0.2 μM MTX on purine and pyrimidine nucleotide pools in MOLT-4, RAJI and KM-3 cells. These effects were correlated with the effects of MTX on cytotoxicity (trypan blue exclusion and soft agar colony forming activity) and cell cycle phase distribution, as determined by DNA-flow cytometry. The differences found in these three cell lines and the significance of an inhibited PDNS with respect to the effects of MTX on (deoxy)ribonucleotide levels are discussed.

MATERIALS AND METHODS

MTX (Emtrexate PF) was purchased from Pharmachemie (Haarlem, The Netherlands); the cell lines MOLT-4 and RAJI have been maintained in continuous culture in our laboratory for several years. KM-3 cells were supplied by the Department of Hematology, St Radboud Hospital, University of Nijmegen (head prof. Dr. C. Haanen). The corresponding cell doubling times are 20 hr for MOLT-4 cells, 20 hr for RAJI cells and 16 hr for KM-3 cells.

The conditions for cell culture, soft agar colony forming activity and DNA-flow cytometry were identical to those described by us earlier [28, 29]. Colony forming activity was expressed as percentage of the plating efficiency of untreated cells. Plating efficiency of untreated cells was (mean ± SD, in %): MOLT-4: 7.0 ± 3.8 (N = 16); RAJI: 35.8 ± 8.9 (N = 15); KM-3: 19.6 ± 6.6 (N = 10).

The assay of purine and pyrimidine nucleotide pools was described earlier by us [29]. The results are expressed as percentages of untreated cells, harvested at the same time of incubation. Initial levels (pmol/10^6 viable cells) of (deoxy)ribonucleotides in untreated cells are shown in Table 1.

RESULTS

Effects of MTX on cell growth and viability, soft agar colony forming activity and cell cycle phase distribution

The assays described below were performed at 0, 24 and 48 hr after addition of MTX and at some points which proved to be important with regard to the time- and dose-dependent inhibitory effects of MTX on PDNS [5]. Therefore, these time intervals are not identical in the three cell lines and differ between the concentrations of MTX used (0.02 μM and 0.2 μM).

The effects of treatment with MTX on cell growth, cell viability (determined by trypan blue exclusion) and on colony forming activity are presented in Figs. 1A and 1B and in Table 2, respectively. After incubation with 0.02 μM MTX cell growth is retarded and even levels off after 20 hr in MOLT-4 cells. Arrest of growth is seen earlier in RAJI and KM-3 cells, but RAJI cells continue to grow, albeit very slowly. The KM-3 cells show a decline in their growth curve. A more significant loss of cell viability (Fig. 1B) is observed in MOLT-4 cells after 48 hr than in RAJI and KM-3 cells.

In MOLT-4 cells clonal growth capacity is impaired in the presence of 0.02 μM MTX (Table 2). However, a significantly increased clonal growth is observed after 72 hr. The explanation for this phenomenon is found in the data from the DNA-flow cytometric studies (Fig. 1C). After incubation with 0.02 μM MTX MOLT-4 cells accumulate in G1 phase and subsequently in early S phase, which can be demonstrated by the continuing broadening of the descending slope of the first histogram peak during the first 28 hr. After 48 hr a second peak in

Table 1. Initial levels of purine and pyrimidine nucleoside triphosphates in human malignant lymphoblasts of different lineages

Cell type		ATP	GTP	UTP	CTP
MOLT-4	(N=6)	1222 ± 92	282 ± 31	449 ± 52	141 ± 21
RAJI	(N=7)	1175 ± 225	264 ± 48	359 ± 99	129 ± 28
KM-3	(N=7)	968 ± 72	276 ± 73	303 ± 24	115 ± 13
		dATP	dGTP	dTTP	dCTP
MOLT-4	(N=6)	23 ± 4	12 ± 2	17 ± 3	7 ± 1
RAJI	(N=7)	8 ± 1	3 ± 0.5	9 ± 1	7 ± 1
KM-3	(N=7)	18 ± 5	24 ± 12	24 ± 7	10 ± 3

MOLT-4: T-cells, RAJI: B-cells; KM-3: non-B-non-T, common ALL cells. N = number of experiments. Results (mean ± SD) are expressed as pmol/10^6 viable cells.

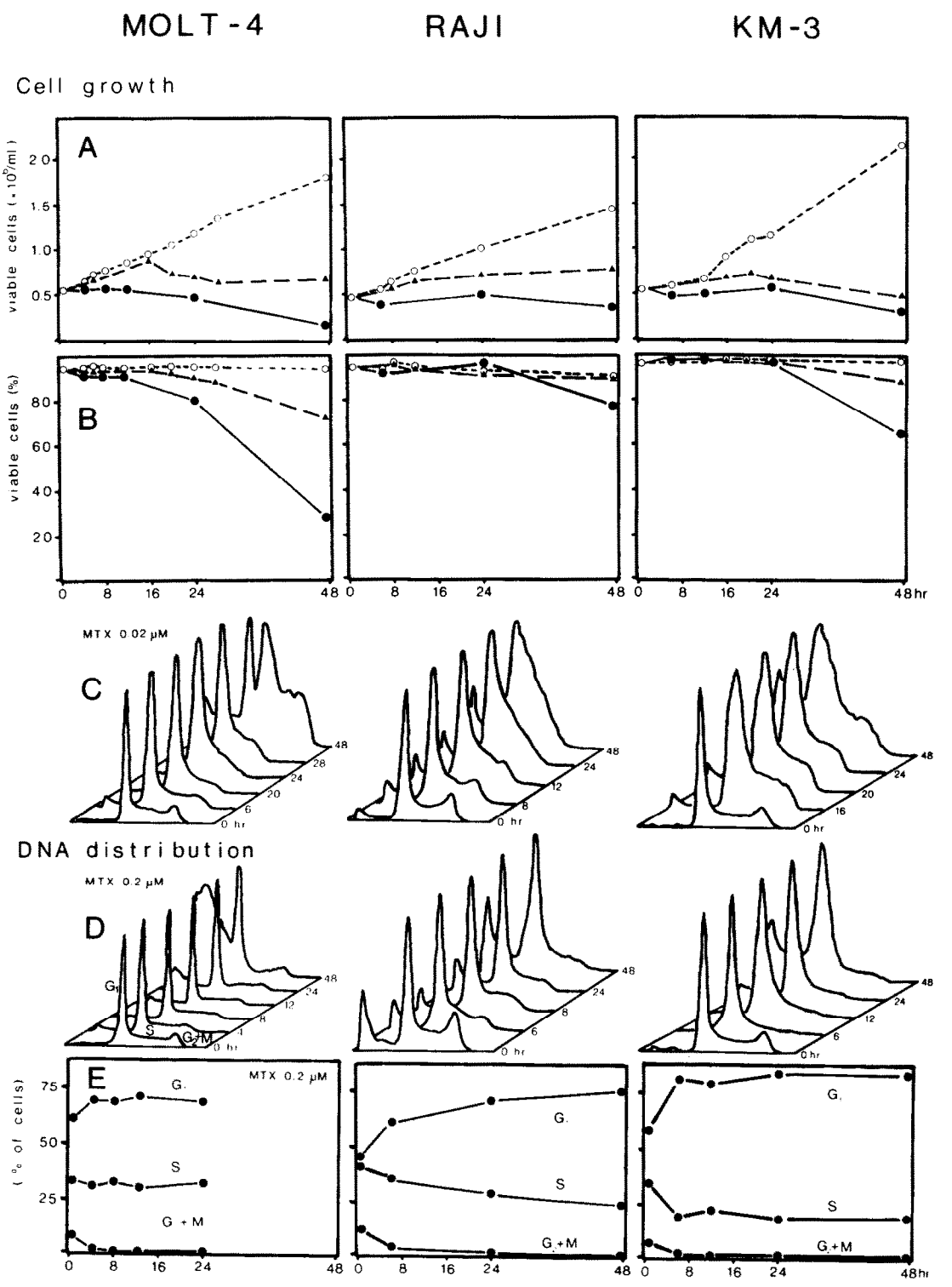

Fig. 1. Effects of MTX on cell kinetic parameters in MOLT-4, RAJI, and KM-3 cells. (A) Effects on cell growth. Counts are expressed as numbers of viable cells only (trypan blue exclusion). The dots represent the mean of at least 3 individual experiments in duplicate. ○, untreated cells; ▲, 0.02 μM MTX; ●, 0.2 μM MTX. (B) Effects of MTX on cell viability, expressed as percentages of viable plus non-viable cells. See further legend to Fig. 1A. (C, D) Effects of MTX on changes in DNA distribution. (C) 0.02 μM MTX; (D) 0.2 μM MTX. The histograms are representative examples of 3 individual experiments. The heights of the G1 peaks were normalized in the graphs. (E) Computer calculations of the percentages of cells in G1, S and G2 + M phase after exposure to 0.2 μM MTX (mean of 3 experiments in duplicate).

Table 2. Effects of MTX on the colony forming activity of human malignant lymphoblasts of different lineages

Duration of incubation (hr)	0.02 μM MTX			0.2 μM MTX		
	MOLT-4	RAJI	KM-3	MOLT-4	RAJI	KM-3
0	100%	100%	100%	100%	100%	100%
6	—	—	—	—	90%	95%
12	—	123%	—	67%	—	—
16	—	—	90%	—	—	—
24	120%	—	—	—	—	—
30	—	—	—	—	28%	12%
36	—	105%	—	2.3%	—	—
40	—	—	6.3%	—	—	—
48	44%	—	—	—	—	—
54	—	—	—	—	4.7%	0.6%
60	—	57%	—	0.05%	—	—
64	—	—	1.1%	—	—	—
72	138%	—	—	—	—	—

Colony forming activity is defined as the percentage of plating efficiency of treated cells/plating efficiency of untreated cells. —, not done.

the S phase of MOLT-4 cells is obvious, indicating a progression of a synchronized cohort of cells through the cell cycle.

RAJI cells show a delayed progression from G1 phase into subsequently early, mid and late S phase (Fig. 1C), demonstrated by the straightening of the descending slope of the G1-S peak, and indicating that a considerable number of RAJI cells continue to progress through the cell cycle. This is associated with only a small decrease of clonal growth in RAJI cells treated with 0.02 μM MTX (Table 2).

KM-3 cells are arrested and synchronized in G1 phase and subsequently in early S phase without further progression through the cell cycle, confirming the most pronounced inhibition of clonal growth with 0.02 μM MTX. It should be noted that exact computer calculations of DNA distribution in cells treated with 0.02 μM MTX were not feasible because of the indistinct separation of the G1 and S peaks and the marked effects on these cell phases.

Incubation with 0.2 μM MTX produces a complete and immediate inhibition of cell growth in all cell lines (Fig. 1A), associated with an increasing cytotoxicity and loss of cell viability, which is most pronounced in MOLT-4 cells (Fig. 1B). This is confirmed by the sharp decrease of clonal growth (Table 2) of MOLT-4 cells treated with 0.2 μM MTX (2.3% of control after 36 hr). Again, RAJI cells are inhibited less. This is also demonstrated by the DNA-flow cytometry results (Figs 1D and 1E), which show an increasing and continuing accumulation of RAJI cells in S1 phase and a continuing decrease of cells in S and G2 + M phase.

In MOLT-4 and KM-3 cells a rapid initial accumulation and arrest in G1 phase is observed, associated with a rapid decrease in S phase in KM-3 cells and in G2 + M phase in both (Figs 1D and E). These events are followed within 8 hr by a complete cessation of cell progression through all parts of the cell cycle in both cell lines, because the percentages of cells in G1 and S phases remain unchanged and cells in G2 + M phase are undetectable. These phenomena are more pronounced in KM-3 cells. DNA distribution data in KM-3 cells at 24 hr show that 82.4% of the cells are in G1, 17.6% in S, and 0% in G2 + M. Corresponding values for MOLT-4 cells are G1: 67.7%; S: 32.3%; G2 + M: 0%. The cytotoxic sensitivity, shown by the extensive decrease of cell viability and clonal growth and the pre-G1 peak of cell debris in Fig. 1D, is more obvious in MOLT-4 cells than in KM-3 cells.

Effects of MTX on purine and pyrimidine nucleotide pools

Deoxyribonucleoside triphosphates. The effects of incubation with MTX on the inhibition of thymidylate synthetase (TS) are shown in Fig. 2. After incubation during up to 16 hr with 0.02 μM MTX a decrease of dTTP levels is apparent in all cell lines (Fig. 2a), although less significant in MOLT-4 cells. dTTP levels in RAJI and KM-3 cells remain depressed, most pronounced in RAJI cells. Incubation with 0.2 μM MTX (Fig. 2b) causes depletion of dTTP levels in all cell lines, again the most pronounced decline is seen in RAJI cells.

The initial decrease of dATP and dGTP levels between 0 and 16 hr in MOLT-4 cells treated with 0.02 μM MTX (Fig. 2a) is followed by an increase of dATP and dGTP and, especially, a more significant increase of dCTP levels at 28 hr. This finding is in agreement with an accumulation and synchronization of cells in early S phase as shown in Fig. 1C, and with results described by others [12, 14, 17, 22, 24, 25, 30–32]. Between 28 and 48 hr dATP and dGTP levels return to the values of untreated cells and dCTP levels decrease, which is associated with a recovery of DNA synthesis and a progression of a cohort of cells through the cell cycle (Fig. 1C) and recovery of clonal growth afterwards (Table 2).

The decrease of dTTP levels in KM-3 cells treated with 0.02 μM MTX (Fig. 2a) is associated with an initial decrease of dATP and dGTP, whereas dCTP levels are unchanged. These events are in agreement with the initial arrest of KM-3 cells in G1 phase (Fig. 1C). However, after 16–24 hr deoxyribonucleotide

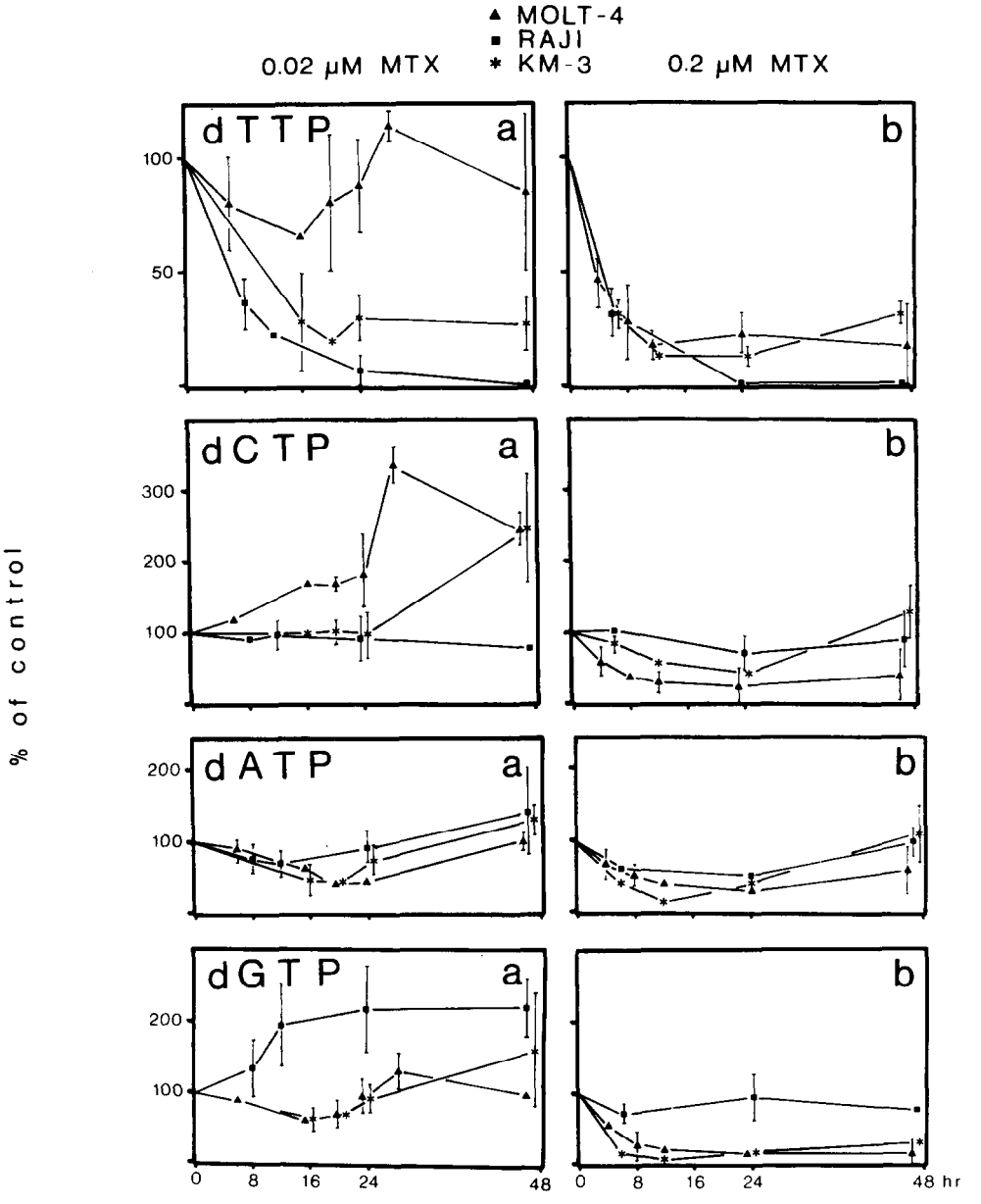

Fig. 2. Changes in dTTP, dCTP, dATP and dGTP levels in MOLT-4, RAJI, and KM-3 cells after exposure to (a) 0.02 μM MTX and (b) 0.2 μM MTX. Results were expressed as percentages of untreated cells at each point of time. Mean ± SD of 3 experiments in duplicate. SD not shown when < 5%.

pools, except for dTTP, show an increase. The pronounced increase of dCTP levels at 48 hr confirms a more obvious arrest and synchronization in early S phase (Fig. 1C), shown by the broadening of the descending slope of the G1-S peak of the histogram. The increase of dCTP levels in KM-3 cells occurs at a later point of time than in MOLT-4 cells, indicating a more delayed progression of KM-3 cells through G1 and early S phase. This is in line with the significant and increasing inhibition of clonal growth in KM-3 cells by 0.02 μM MTX in Table 2.

The changes in deoxyribonucleotide pools in RAJI cells treated with 0.02 μM MTX differ in many aspects from those in MOLT-4 and KM-3 cells (Fig. 2a). dTTP levels are severely depleted; however, dCTP levels are almost unchanged. dATP levels decrease to a minimum of 67% at 12 hr, followed by increasing values. However, dGTP levels show an increase. These events are associated with a delayed, but substantial progression through the cell cycle shown in Fig. 1C and confirm the minor inhibition of cell growth and clonal growth in RAJI cells treated with 0.02 μM MTX.

The effects of incubation with 0.2 μM MTX on deoxyribonucleotide pools are presented in Fig. 2b. The decrease of dTTP, dATP and dGTP levels is almost similar in MOLT-4 cells and KM-3 cells, although KM-3 cells are more affected. These events

are associated with an initial increase and synchronization of cells in G1 phase followed by a complete cessation of cell progression through the cell cycle (Figs. 1D and 1E), which is again more pronounced in KM-3 cells. The increasing severe inhibitions of clonal growth in MOLT-4 and KM-3 cells treated with 0.2 μM MTX (Table 2) are in agreement with these phenomena. At 48 hr an increase of deoxyribonucleotide pools, except for dTTP, is noticed, due to reutilization of purine and pyrimide nucleotide precursors, without restoration of DNA synthesis [29].

The relatively small initial decrease of dATP, dGTP, and dCTP (Fig. 2b) in RAJI cells after treatment with 0.2 μM MTX is in accordance with the increasing accumulation of cells in G1 phase and a decrease of cells in S and G2 + M phase. However, a small proportion of cells is still able to progress slowly through the cell cycle, as demonstrated by the data of DNA distribution (Fig. 1D) at 48 hr (G1: 74.9%; S: 24.7%; G2 + M: 0.4%) and the incomplete inhibition of clonal growth (Table 2). The relatively unchanged dGTP pools in RAJI cells are a conspicuous phenomenon.

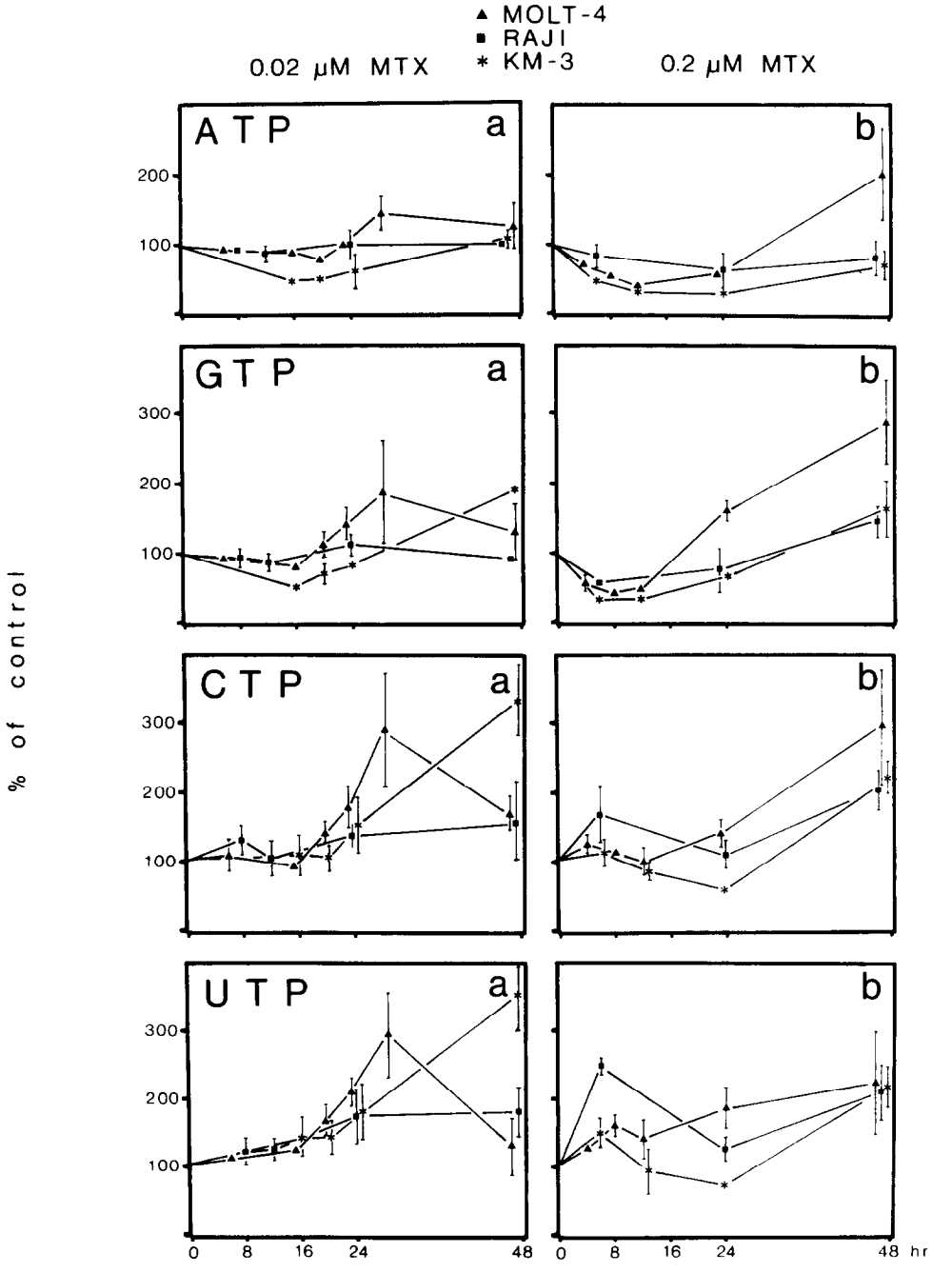

Fig. 3. Changes in ATP, GTP, CTP and UTP levels in MOLT-4, RAJI, and KM-3 cells after exposure to (a) 0.02 μM MTX and (b) 0.2 μM MTX. See further legend to Fig. 2.

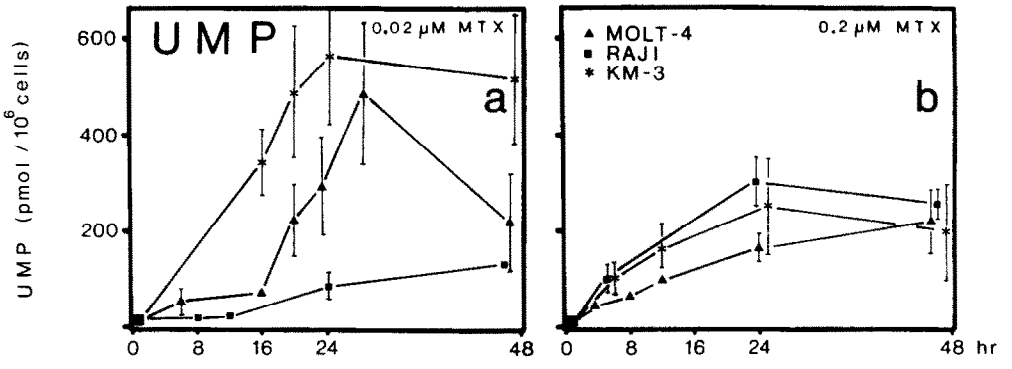

Fig. 4. Changes in UMP levels in MOLT-4, RAJI, and KM-3 cells after exposure to (a) 0.02 μM MTX and (b) 0.2 μM MTX. Mean ± SD of 3 experiments in duplicate. Expressed as pmol/10^6 viable cells. SD not shown when < 20 pmol.

Ribonucleoside triphosphates. The changes in ribonucleoside triphosphate pools and UMP after treatment with MTX are demonstrated in Figs 3 and 4. Levels of ribonucleoside mono- and diphosphate pools were assayed simultaneously, but the data are not shown here because of the less significant differences, except for UMP. During the first 16 hr of treatment with 0.02 μM MTX (Figs 3a and 4a) a small initial decrease of ATP and GTP levels is associated with an increase of UTP, CTP and UMP levels. These phenomena coincide with a partial inhibition of PDNS, an increasing availability of PRPP, an increased pyrimidine *de novo* synthesis and consumption of ribonucleotides for RNA synthesis [5, 16, 23]. After 16 hr an increase of all ribonucleotide levels is observed, especially in MOLT-4 and KM-3 cells. This suggests a cessation of consumption for RNA synthesis due to maximal inhibition of RNA synthesis.

At 48 hr pyrimidine ribonucleotide pools in MOLT-4 cells treated with 0.02 μM MTX decrease. This may be due to a decrease of PRPP levels by restoration of PDNS [5] or recovery of RNA synthesis with consumption of ribonucleotides. However, the decrease of purine ribonucleotides indicates that the latter is the case in MOLT-4 cells, as was also shown in the T-cell line CCRF-CEM treated with 0.02 μM MTX [21]. In KM-3 cells all ribonucleotide pools increase further at 48 hr, indicating an ongoing inhibition of RNA synthesis. The effects of 0.02 μM MTX on ribonucleotide synthesis and RNA synthesis are most pronounced in KM-3 cells. These cells also displayed the most pronounced inhibition of purine *de novo* synthesis [5]. In RAJI cells the changes in ribonucleotide pools are less (Fig. 3a) and UMP levels (Fig. 4a) only increase moderately, suggesting almost no impairment of RNA synthesis. RAJI cells demonstrated a relatively low activity of PDNS and a high activity of the purine salvage pathway [5], which permits these cells to escape from purine depletion due to MTX treatment.

Treatment with 0.2 μM MTX induces an initial depletion of purine ribonucleotides (Fig. 3b) in all cell lines due to complete inhibition of PDNS [5] and consumption for an ongoing, but decreasing RNA synthesis [23]. The initial increase in pyrimidine ribonucleoside triphosphate pools with maxima at 6 hr for RAJI and KM-3 cells and at 8 hr for MOLT-4 cells is exactly correlated with the maximal levels of PRPP [5]. Thereafter, all ribonucleoside triphosphate pools decrease, leading to a complete inhibition of RNA synthesis at 12 hr in MOLT-4 cells, and at 24 hr in RAJI and KM-3 cells. The increase of ribonucleoside triphosphates at 48 hr is a consequence of reutilization of purine and pyrimidine precursors [16, 23, 33–35] from increased cell kill and nucleic acid breakdown. This is most obvious in MOLT-4 cells, in which cytotoxicity is most pronounced (Figs. 1A and 1B and Table 2). However, the increased levels of ribonucleoside triphosphates are not consumed for RNA synthesis and do not result in a restoration of RNA synthesis as was shown in MOLT-4 cells treated with 0.02 μM MTX.

DISCUSSION

Untreated MOLT-4 (T-), RAJ (B-) and KM-3 (non-B-non-T, common ALL) cells demonstrate important differences with regard to their purine and pyrimidine metabolism and cell-kinetic parameters. Our time-dependent measurements after exposure to MTX clearly demonstrate a correlation between these differences in untreated cells and the effects of MTX on metabolic and cell-kinetic parameters. These differences were most obvious in incubation experiments with the critical concentration 0.02 μM MTX. They can be attributed to the characteristics of the cell lines and their connection with the biochemical basis for MTX toxicity [36–41].

Although the cell doubling times of the three cell lines were similar, our DNA-flow cytometric studies demonstrated that the percentages of untreated cells in S phase and G2 + M phase were significantly higher in RAJI cells than in MOLT-4 and KM-3 cells (Table 3), indicating a longer duration of these cell cycle phases in RAJI cells. The long duration of these cell cycle phases is reflected in the relatively low pools of deoxyribonucleotides in RAJI cells (Table 1), as was shown earlier in other B-cell lines [42]. These data may suggest that the rate of deoxyribonucleotide consumption for DNA synthesis in RAJI cells is relatively high compared with the rate of deoxyribonucleotide supply by the S phase-specific enzymes thymidylate synthetase (TS) and ribo-

Table 3. Distribution of cells in various cell cycle phases, as determined by DNA-flow cytometry

		G1	S	G2 + M
MOLT-4	(N=53)	57.4 ± 3.0	35.7 ± 3.1	6.8 ± 2.2
RAJI	(N=27)	44.9 ± 8.9	40.7 ± 2.7	12.5 ± 2.1
KM-3	(N=13)	58.3 ± 3.0	34.8 ± 2.4	7.2 ± 2.0

Mean ± SD, expressed as percentages of cells; N = number of experiments.

nucleotide reductase [30–32, 39, 40, 43, 44]. The low cytotoxicity in RAJI cells, shown in Table 2, might be correlated with an increased length of the S phase of the cell cycle. Although the S phase is the target of MTX [45–52], Hill [53] demonstrated that treatment with the antifolate metoprine did not delay the progression of synchronized late S phase cells through the cell cycle, indicating that only early and mid S phase cells are sensitive to MTX.

The possible role of polyglutamation of MTX with respect to the differences in inhibition of PDNS between the three cell lines was discussed in our previous study [5]. The increase of clonal growth activity after 72 hr in MOLT-4 cells exposed to 0.02 μM MTX (Table 2), the reversal of the partial inhibition of PDNS [5] and the minor effects on dTTP levels in MOLT-4 cells in the presence of 0.02 μM MTX (Fig. 2a), suggest that this concentration of MTX is unable to result in formation of MTX polyglutamates in MOLT-4 cells [54]. The increasing depletion of dTTP levels in RAJI and KM-3 cells indicate an in time increasing inhibition of TS by increasing levels of MTX polyglutamates, which are more potent and direct inhibitors of TS [41, 55, 56]. Our data in Table 2 and the inhibition of PDNS [5] confirm the contribution of increasing amounts of MTX polyglutamates, especially in KM-3 cells. However, these data also demonstrate the limitations of the soft agar colony assay in predicting MTX-toxicity. As shown in Table 2, colony forming activity of MOLT-4 and RAJI cells, incubated with 0.02 μM MTX, was higher than 100% at a considerable number of time-points, whereas other assays indicate an inhibition. This has to be attributed to efflux of "free", non-polyglutamated MTX during the wash-procedure, before the cells are allowed to grow in fresh agar medium [41, 57–62]. We did not notice controversial results from our soft agar assays, when the changes in dTTP levels and the inhibition of PDNS indicated the presence of MTX polyglutamates. Polyglutamates are preferentially retained by cells and will not be washed out during the soft agar procedure [41, 60–62].

We demonstrated the prominent role of the activity of the PDNS and the purine salvage pathway, especially with respect to cytotoxicity of 0.02 μM MTX. Cytotoxicity (Table 2) was rather limited in RAJI cells with a relatively low PDNS and a high salvage activity [5]. This could be attributed to a partial inhibition of PDNS, whereas the purine salvage pathway was able to supply the cells with essential purine and pyrimidine precursors, for example from RNA turnover. The partial inhibition of PDNS in RAJI cells in the presence of 0.02 μM MTX did not impair ATP and GTP pools (Fig. 3a). In MOLT-4 cells, with both pathways active, cytotoxicity was the least, since the inhibition of PDNS was almost absent, and the cells also could rely on the activity of the purine salvage pathway for their supply. However, KM-3 cells with both pathways moderately active, demonstrated the highest cytotoxicity of 0.02 μM MTX. The purineless state of KM-3 cells was a consequence of the severe inhibition of PDNS and the inability of the rather low purine salvage pathway to supply the cells with purines. The pools of ATP and GTP in Fig. 3a confirmed the purineless state in KM-3 cells. These data indicate that low concentrations of MTX can introduce a purineless state in cells which rely partially on the salvage pathway for their purine supply, but also on their moderately active PDNS. However, when the activity of the salvage pathway is high (RAJI cells), a purineless state is prevented.

The low activity of PDNS in bone marrow cells and lymphocytes, which escape from MTX toxicity by their active salvage pathway, again elucidates the selective toxicity of MTX for malignant cells. This escape is also favoured by the fact that normal cells, like bone marrow and small intestinal cells, have a low capacity for glutamation of MTX [41, 62–64]. When MTX exposure is prolonged, or when MTX doses are extremely high, non-polyglutamated MTX can be toxic for rapidly growing normal cells due to a prolonged thymidylateless state. However, this offers protective capacities for normal cells, when thymidine and a purine are administered early after MTX [65].

The role of a purineless state with regard to the toxic effects of MTX has been the object of intensive study in the literature. Many authors based their conclusions on studies showing the reversal of MTX cytotoxicity by addition of purine bases alone, thymidine alone or a combination of a purine plus thymidine [33, 35, 51, 66–74]. Controversial results were obtained with these "reversal" studies between different cell lines, different investigators and even within one study. These controversial conclusions with regard to the role of the purineless or the thymidylateless state have to be attributed to the fact that addition of these precursors (hypoxanthine, thymidine, uridine, deoxyuridine, deoxycytidine) may change the biochemical disturbances of MTX on both the purine and the pyrimidine pathways. Moreover, treatment with MTX may alter the activity of enzymes involved in the conversion of these precursors [36, 37, 71, 75].

Our data indicate that combined measurements of all nucleotide pools, the activity of PDNS and cell-kinetic studies, especially DNA-flow cytometry, are necessary in order to understand the cytotoxic effects

of MTX. This is stressed by the perturbations of the dTTP pools in our cell lines. Although MTX caused a severe depletion of dTTP levels in RAJI cells, our cell-kinetic studies showed the least effects in these cells (Table 2, Fig. 1). Thus, these results failed to demonstrate the main role of the inhibition of TS with respect to MTX toxicity, as has been proposed by others [33, 51]. Our study showed the most severe impairment of cell growth and DNA-distribution, when both dTTP and dGTP pools were severely depleted, as was the case in KM-3 cells with both concentrations of MTX and in MOLT-4 cells after incubation with 0.2 μM MTX. Under these circumstances we noticed a complete inhibition of DNA synthesis, PDNS and RNA synthesis (balanced growth pattern). These phenomena confirm the findings of Taylor *et al.* [25, 26].

MOLT-4 and RAJI cells, incubated with 0.02 μM MTX demonstrated a "classical" unbalanced growth pattern (partial inhibition of DNA synthesis and less inhibition of PDNS and RNA synthesis [76]), whereas treatment with 0.2 μM MTX in RAJI cells completely inhibited PDNS and RNA synthesis, but allowed some progression of cells through the cell cycle. Under these circumstances MTX treatment did not impair both dTTP and dGTP levels and cytotoxicity was rather low.

The differences between MOLT-4, RAJI and KM-3 cells may have consequences for further studies in patients with T-, B- and non-B-non-T-, common-ALL. Our data suggest that maintenance therapy with low dose MTX (0.02 μM) can be effective in KM-3 cells, since these cells are able to form polyglutamates, which was denied by others [38]. However, low dose MTX therapy does not result in sufficient cytotoxicity in MOLT-4 and RAJI cells. High dose MTX therapy, is able to result in severe cytotoxicity in all cell lines; however, RAJI cells may recover. These data might be transferred to the *in vivo* situation: Treatment of common ALL with maintenance therapy, including low dose MTX and 6-mercaptopurine, has proved to result in superior survival as compared to T-cell ALL. The current therapy of B-cell lymphoma and B-cell ALL [3] with short intensive courses including high-dose MTX at short intervals without maintenance therapy has dramatically improved survival in these patients.

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