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DOSE-RELATED EFFECTS OF METHOTREXATE ON PURINE AND PYRIMIDINE NUCLEOTIDES AND ON CELL-KINETIC PARAMETERS IN MOLT-4 MALIGNANT HUMAN T-LYMPHOBLASTS*

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Abstract—The effects of methotrexate (MTX) on cytotoxicity (trypan blue exclusion and soft agar clonal growth), cell cycle perturbation, and purine and pyrimidine ribonucleotide and deoxyribonucleotide pools have been studied in MOLT-4 malignant T-lymphoblasts. Two concentrations of MTX, 0.02 μM and 0.2 μM have been utilized, which can be maintained *in vivo* during many hours in the maintenance therapy of acute lymphoblastic leukemia (ALL). The results are correlated with the effects of MTX on the inhibition of purine *de novo* synthesis. Treatment with 0.02 μM MTX results in an accumulation of cells in early S phase after 20 hr, as measured by DNA flow cytometry and by a significant increase of dCTP levels, followed by a slow progression of a cohort of cells through the cell cycle. Cytotoxicity also becomes evident starting from this point of time. The effects on deoxyribonucleotide pools are discussed in correlation with the inhibition of DNA synthesis. The changes in ribonucleotide pools are associated with the partial inhibition of purine *de novo* synthesis at 20–28 hr and suggest an inhibition of RNA synthesis. After 48 hr a reutilization of nucleotide precursors due to nucleic acid breakdown and a recovery of purine *de novo* synthesis is shown, associated with a recovery of RNA synthesis, whereas cytotoxicity increases. Treatment of MOLT-4 cells with 0.2 μM MTX results in a rapid complete cessation of cell progression through all parts of the cell cycle after 8 hr, associated with a depletion of all deoxyribonucleotide pools, complete inhibition of purine *de novo* synthesis, inhibition of RNA synthesis and a marked cytotoxicity. Ribonucleotide pools demonstrate a reutilization of nucleotide precursors after 12 hr of incubation without a recovery of purine *de novo* synthesis and RNA synthesis.

These data show a close dose- and time-dependent correlation of the effects of MTX on purine *de novo* synthesis, UMP levels and other (deoxy)ribonucleotide pools, and on RNA and DNA synthesis in MOLT-4 cells having an active purine *de novo* synthesis. This correlation is absent in normal bone marrow cells and peripheral blood lymphocytes. These data can be used in order to elucidate the synergistic effects of sequential administration of MTX and 6-mercaptopurine.

Methotrexate (MTX) is a potent drug in the treatment of acute lymphoblastic leukemia (ALL), used either as high dose intravenous chemotherapy and prophylactic central nervous system treatment [1], or as oral maintenance treatment in combination with oral 6-mercaptopurine (6MP) [2].

We demonstrated a sequence-, time- and dose-dependent synergism of MTX and 6MP in MOLT-4 malignant human T-lymphoblasts [3]. The increased availability of 5-phosphoribosyl-1-pyrophosphate (PRPP) after pretreatment with MTX could be used for an enhanced conversion of 6MP by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). This is a selective phenomenon in malignant lymphoblasts with an active purine *de novo* synthesis and is absent in normal bone marrow cells and peripheral blood lymphocytes [3, 4].

Determination of aminoimidazolecarboxamide ribonucleotide (AICAR) levels and labeled glycine incorporation studies indicated a complete inhibition of purine *de novo* synthesis after treatment with 0.2 μM MTX and an incomplete inhibition with 0.02 μM MTX in MOLT-4 lymphoblasts [3].

Extensive studies [5–19] have been published with respect to the effects of various concentrations of MTX on cytotoxicity, cell cycle perturbation and purine and pyrimidine deoxyribonucleotide pools in various malignant cell lines. However, studies concerning these effects of MTX in correlation with the inhibition of purine *de novo* synthesis and ribonucleotide pools are scarcely available.

To our knowledge, the malignant lymphoblastic cell lines currently in study in our laboratory: MOLT-4 T-, Raji B- and KM-3 non-B-non-T-lymphoblasts, have not been studied before, utilizing MTX-concentrations which can be maintained *in vivo* during many hours in the oral maintenance therapy of ALL in children [20–22].

In the present study we investigated the sequential time-dependent effects of 0.02 μM and 0.2 μM MTX on purine and pyrimidine nucleotide pools in MOLT-4 cells. These effects were correlated with the effects of MTX on cytotoxicity (trypan blue exlcusion and soft agar colony forming activity) and cell phase distribution, measured by DNA-flow cytometry. The significant role of the inhibition of purine *de novo*

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synthesis with regard to the effects of MTX on (deoxy)ribonucleotide levels and DNA and RNA synthesis is discussed.

MATERIALS AND METHODS

Materials. MTX (Emtrexate PF) was purchased from Pharmachemie, Haarlem, The Netherlands; MOLT-4 human malignant T-lymphoblasts from Flow Laboratories, Irvine, U.K.; Bacto-agar from Difco Laboratories, Detroit, MI.

Cell culture. The conditions for cell culture were similar as described earlier [3].

Soft agar colony forming activity. Before cloning, cells were washed once in fresh medium, centrifuged (5 min, 200 *g*) and resuspended in medium in order to ensure drug removal from the medium. Cell count and viability were measured. Two ml suspensions containing cells and Bacto-agar 0.3% (w/v) in fresh medium were layered into 6-wells plates (Becton Dickinson, Oxnard, CA). Colonies were allowed to grow at 37° in a water-saturated atmosphere containing 2.5% CO_2. After 10 days colonies of at least 30 cells were counted, using a Leitz Diavert microscope. Cloning results were expressed as plating efficiency (in %): number of colonies × 100/number of cells before plating. In these experiments we adjusted the cell concentrations in order to obtain a linear correlation between number of cells before plating and number of colonies. Therefore, we diluted the cells after washing to three known concentrations in duplicate and chose for each point the highest plating efficiency found. Plating efficiency of untreated MOLT-4 cells was 8%.

Colony forming activity was expressed as percentage of plating efficiency of untreated cells. All experiments were performed in duplicate.

DNA-flow cytometry. At each point of time 4 ml of the cell suspension were centrifuged (8 min, 400 *g*, 4°). Cell pellets were resuspended in 0.2 ml NaCl 0.9% solution and 1.8 ml hypotonic ethidium bromide solution, containing 25 mg/l ethidiumbromide and 1 g/l sodium citrate. Distributions of DNA content were analyzed in an impulse cytophotometer (ICP 11; Phywe Company, F.R.G.), and calculated utilizing the method described by Baisch *et al.* [23]. The results were expressed as percentages of cells in G1, S, and G2 + M phase. In the computerised three-dimensional graphics the G1 peak is placed in channel 50 on the abscissa and the heights of all G1 peaks were normalized. Exact computer calculations of DNA distribution in cells treated with 0.02 μM MTX were not feasible with this procedure because of the indistinct separation of the G1 and S peaks.

DNA distribution of untreated cells was (mean ± SD, N = 6): G1: 60.3 ± 3.5, S: 32.3 ± 2.4, G2 + M: 7.7 ± 0.8.

Assay of purine and pyrimidine nucleotide pools. After centrifugation 100 μl 0.4 M $HClO_4$ was added to the cell pellet, containing approximately 44 × 10^6 cells, and kept on ice during 15 min. The suspension was centrifuged (4 min, 5400 *g*, 4°), the supernatant was adjusted to a pH range of 6.0–6.7 with a solution containing 1 M K_2HPO_4 + 0.4 M KOH + phenol-red, and centrifuged again. 1/11 fraction of the supernatant was utilized for determination of ribonucleotides. For determination of deoxyribonucleotides we had to oxidize the ribonucleotides. The remaining 10/11 fraction of the supernatant was adjusted to 150 μl and we added 6 μl 0.5 M sodium periodate and 7.5 μl 4.0 M diethylaminephosphate (pH 7.5). After 30 min of incubation at 37° 2 μl 1 M rhamnose was added to the mixture in order to neutralize the remaining sodium periodate and the samples were put on ice and measured immediately afterwards. Chromatography was performed on an HPLC, model SP 8000 (Spectra Physics, Santa Clara, CA) with an automated data system and a fixed wavelength u.v.-detector of 254 nm. Separation was carried out at 45° on a Partisil-10-SAX column (250 × 4.6 mm; Whatman, Maidstone, U.K.) with a mobile phase consisting of three elution buffers, as described in Table 1: (A) 0.05 M potassium dihydrogenphosphate + 2% (v/v) CH_3CN, pH = 3.10; (B) 2% (v/v) CH_3CN in water, and (C) 0.45 M potassium dihydrogenphosphate + 2% (v/v) CH_3CN, pH = 5.25. During the elution the solutions were deaerated by continuous helium purging. A constant flow rate of 2.8 ml/min was used.

Table 1. Percentages of buffers* used during elution of ribonucleotides and deoxyribonucleotides

Time (min)	Buffer A (%, v/v)	Buffer B (%, v/v)	Buffer C (%, v/v)
0.0	10.0	90.0	0.0
6.5	60.0	40.0	0.0
13.0	44.0	44.0	12.0
16.2	40.0	40.0	20.0
32.0	30.0	30.0	40.0
48.0	20.0	20.0	60.0
51.0	14.0	14.0	72.0
57.5	14.0	14.0	72.0

* See Materials and Methods.

Recovery, sensibility and reproducibility of the cell extraction procedure were described earlier [24]. However, the procedure described here resulted in lower detection limits. Because we found a decrease of (deoxy)ribonucleotide levels in untreated cells after prolonged periods of incubation, the results (pmol/10^6 viable cells) were expressed as percentages of untreated viable cells, harvested at the same time of incubation.

Initial levels of (deoxy)ribonucleotides in MOLT-4 cells (pmol/10^6 viable cells ± SD, N = 6) were: ATP: 1222 ± 92, GTP: 282 ± 31, UTP: 449 ± 52, CTP: 141 ± 21, dATP: 23 ± 4, dGTP: 12 ± 2, dTTP: 17 ± 3, dCTP: 7 ± 1.

RESULTS

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

Figures 1a and 1b show the effects on cell growth and cell viability, respectively, as determined by trypan blue exclusion, in MOLT-4 cells during incubation with 0.02 μM and 0.2 μM MTX, respectively.

Cell growth is completely inhibited 20 hr after incubation of MOLT-4 cells with 0.02 μM MTX (Fig.

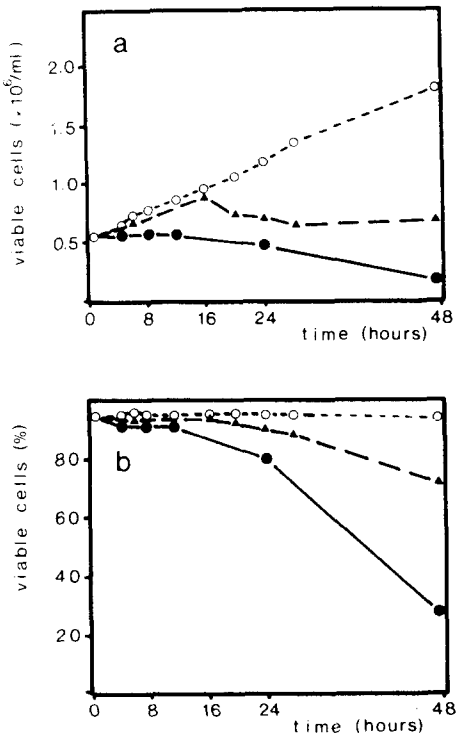

Fig. 1. (a) Effects of MTX on the growth of MOLT-4 cells. Counts are expressed as number of viable cells only (trypan blue exclusion): ○, untreated cells; ▲, 0.02 μM MTX; ●, 0.2 μM MTX. The dots represent the mean of 3 individual experiments in duplicate. (b) Effects of MTX on viability of MOLT-4 cells, expressed as percentages of viable plus non-viable cells. See further legend to Fig. 1a.

1a), and cell viability (trypan blue exclusion) is gradually decreasing (70% at 48 hr, Fig. 1b). However, colony forming activity is not impaired by 0.02 μM MTX at 24 hr (Table 2). This may be the result of washing out the low concentration of MTX with fresh medium before the cells were plated on agar and were allowed to grow for 10 days [12, 17]. The bias of the procedure of soft agar clonal growth due to washing out of MTX has to be taken into account, when the results of *in vitro* and *in vivo* studies with low concentrations of MTX are compared. This phenomenon was not obvious 48 hr after incubation with 0.02 μM MTX, where colony forming activity is reduced to 43.8% (Table 2). Incubation with 0.2 μM MTX produces an inhibition of cell growth already after 4 hr (Fig. 1a), with an increasing loss of cell viability (Fig. 1b). This is reflected in a marked decrease of clonal growth, observed already at 12 hr, the shortest time interval measured (Table 2). An almost complete inhibition of clonal growth is observed at 36 and 60 hr after incubation.

Table 2. Effects of MTX on the colony forming activity* of MOLT-4 T-lymphoblasts

Duration of incubation (hr)	0.02 μM MTX	0.2 μM MTX
0	100.0%	100.0%
12	—	67.2%
24	119.9%	—
36	—	2.25%
48	43.8%	—
60	—	0.05%

* Colony forming activity is defined as the percentage of plating efficiency of treated cells/plating efficiency of untreated cells (see Materials and Methods).
—, not done.

The effects of MTX on cell cycle phase distribution are presented in Fig. 2. After incubation with 0.02 μM MTX a decrease of the percentage of cells in G2 + M phase is noted (Fig. 2a) together with an accumulation of cells in early S-phase shown by the continuing broadening of the descending slope of the first histogram peak during the first 28 hr of incubation. At 48 hr a second peak in the S phase appears, indicating a slow progression of a cohort of cells through the cell cycle. This was also suggested by the percentages of viable cells and the colony forming activity at 48 hr, which show that a considerable number of cells escape from the cytotoxic effects of 0.02 μM MTX.

Incubation with 0.2 μM MTX (Figs. 2b and 2c) demonstrates a rapid initial increase of cells in G1 phase, associated with an almost complete loss of cells in G2 + M phase. After 8 hr of incubation this phenomenon is followed by a complete cessation of cell proliferation through all parts of the cell cycle, which is associated with a significant decrease of cell growth and cell viability (Fig. 1) and of clonal growth (Table 2). The conspicuous decrease of viability at 48 hr, shown by the first peak of cell debris in the histogram preceding the G1 peak, did not allow us to make exact calculations of DNA-distribution at that point of time.

Effects of MTX on purine and pyrimidine nucleotide pools

MTX inhibits tetrahydrofolate dependent enzymes, due to inhibition of dihydrofolate reductase [25, 26]. In pyrimidine metabolism, the inhibition of thymidylate synthetase in MOLT-4 cells after incubation with 0.2 μM MTX is shown by a rapid decrease of dTTP levels (to 20% of untreated cells, Fig. 3b). The purine deoxyribonucleotides, dATP and dGTP (Fig. 4b), and dCTP levels (Fig. 3b) also demonstrate a rapid decrease.

These phenomena explain the complete inhibition of progression through all parts of the cell cycle, shown in Figs 2b and 2c. The complete inhibition of purine *de novo* synthesis due to incubation with 0.2 μM MTX is shown in an initial decrease of ATP and GTP (Fig. 5b). A concomitant initial increase of pyrimidine ribonucleotides is demonstrated (Fig. 6b), which is followed by an increase of all ribonucleotides after prolonged incubation with 0.2 μM MTX. These phenomena and the slow increase of UMP levels (Fig. 7b) will be discussed later.

The effects of 0.02 μM MTX on purine and pyrimidine pools are less pronounced. The partial inhibition of thymidylate synthetase is reflected in a small

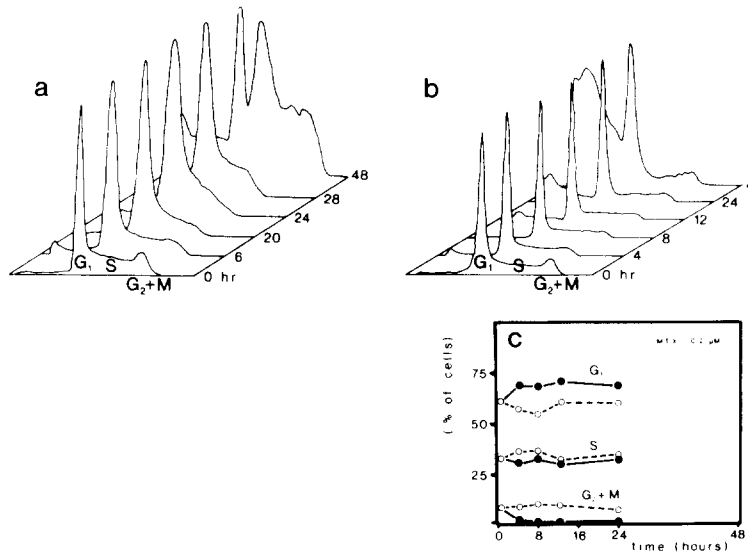

Fig. 2. Effects of MTX with time of exposure on changes in DNA distribution of MOLT-4 cells. (a) 0.02 μM MTX; (b) 0.2 μM MTX. The histograms are representative examples of 3 individual experiments. (c) Computer calculations of the percentages of cells in various cell cycle phases: ○, untreated cells; ●, 0.2 μM MTX. The dots represent the mean of 3 experiments in duplicate.

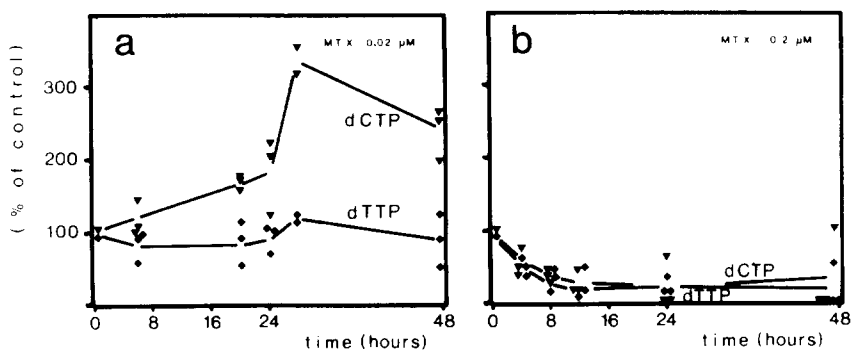

Fig. 3. Changes in dCTP (▼) and dTTP (◆) levels in MOLT-4 cells after exposure to MTX: (a) 0.02 μM MTX; (b) 0.2 μM MTX. Results were expressed as percentages of untreated cells at each point of time. Each dot represents a determination in duplicate. The curves are drawn through the mean of the determinations at each point of time.

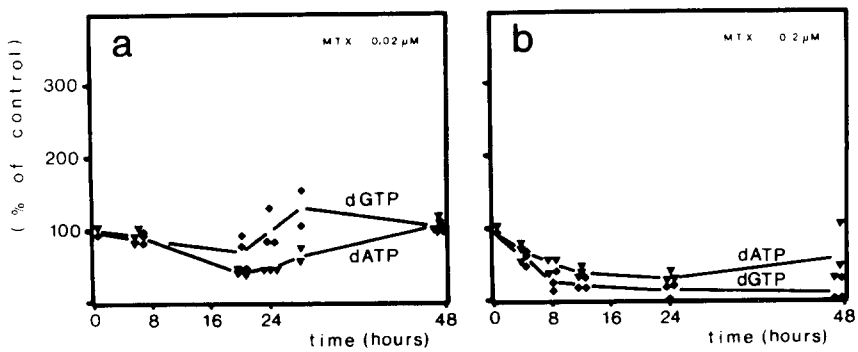

Fig. 4. Changes in dATP (▼) and dGTP (◆) levels in MOLT-4 cells after exposure to MTX. See further legend to Fig. 3.

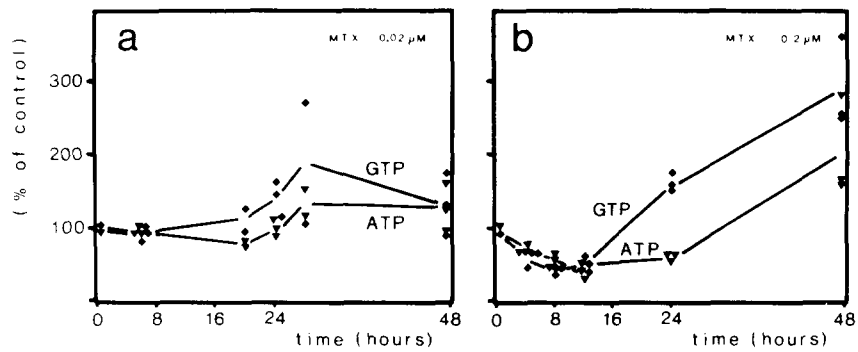

Fig. 5. Changes in ATP (▼) and GTP (◆) levels in MOLT-4 cells after exposure to MTX. See further legend to Fig. 3.

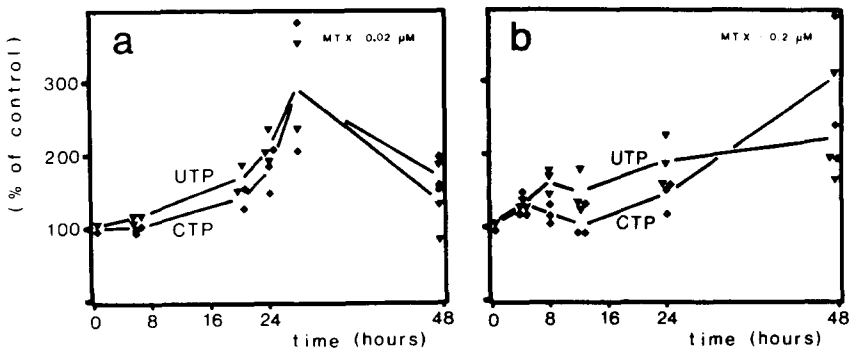

Fig. 6. Changes in CTP (▼) and UTP (◆) levels in MOLT-4 cells after exposure to MTX. See further legend to Fig. 3.

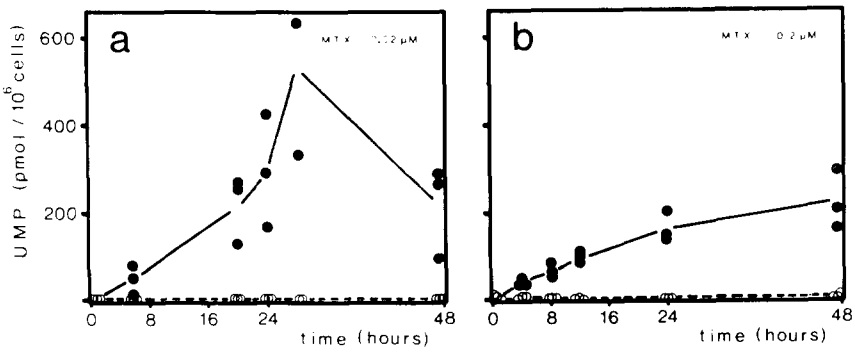

Fig. 7. Changes in UMP levels in MOLT-4 cells after exposure to MTX: (a) 0.02 μM MTX; (b) 0.2 μM MTX; ○, untreated controls; ●, MTX-treated cells. Results are expressed as pmol/10^6 viable cells. See further legend to Fig. 3.

decrease of dTTP levels (Fig. 3a). During the first 20 hr of incubation purine deoxyribonculeotides show a small decrease, which is followed by an increase (Fig. 4a). These effects were associated with an accumulation of cells in early S phase and an increase of dCTP levels (Fig. 3b).

Determinations of PRPP and AICAR [3] demonstrated that a partial inhibition of purine *de novo* synthesis was obtained after 20–28 hr of incubation with 0.02 μM MTX. At those points of time a concomitant increase of UMP (Fig. 7a) and all ribonucleoside triphosphate pools (Figs. 5a and 6a) is noted. It should be mentioned that the assay conditions for determination of UMP do not allow a distinct separation of UMP and oxidized dUMP. However, the course of the levels in Fig. 7 is identical to that of UDP and CDP levels (data not shown) and those of pyrimidine triphosphate levels. Although treatment with high concentrations of MTX (10 μM) resulted in an extensive increase of dUMP [8], the

Table 3. Summary of events as a result of the action of MTX on MOLT-4 cells

	Time (hr) at which the events become evident. 0.02 μM MTX	0.2 μM MTX
Inhibition of cell growth	20	4–8
Inhibition of DNA synthesis	20	8
Maximal increase of PRPP levels	20–28	8
Maximal increase of AICAR levels	28	—
Maximal decrease of purine ribonucleotide levels	—	8–12
Maximal increase of pyrimidine ribonucleotide levels	28	8
Inhibition of RNA synthesis	28	12
Reutilization of nucleotide precursors	48	12
Recovery of purine *de novo* synthesis	48	—
Recovery of RNA synthesis	48	—

— not present.

effects of 0.02 μM and 0.2 μM MTX in our experiments on UDP and UTP levels suggest a predominant contribution of UMP in our assays.

At 48 hr of incubation with 0.02 μM MTX all ribonucleotides decrease to levels of untreated cells, whereas at the same time PRPP and AICAR levels decreased also, suggesting a recovery of RNA synthesis.

Table 3 summarizes the effects of both concentrations of MTX on the parameters mentioned above.

DISCUSSION

The effects of MTX on cell growth, cell kinetics and purine and pyrimidine pools have been studied extensively in various cell lines [5–19]. Excellent reviews of the literature with respect to the various effects of MTX were presented by Jackson [25, 26]. Important differences were demonstrated between different cell lines and, within one cell line, between different investigators. Some investigators used relatively high concentrations of MTX in their cell cultures, up to 10 or 100 μM, which can be obtained with high dose MTX infusions in patients [1]. However, the purpose of our investigations was to study the effects of MTX in concentrations, which can be maintained *in vivo* during many hours in the oral maintenance therapy of ALL in children [20–22], and, moreover, to study the effects of various combinations of MTX and 6MP.

We demonstrated the inhibitory effects of 0.02 μM and 0.2 μM MTX on purine *de novo* synthesis of MOLT-4 cells [3]. These effects were time- and dose-dependent. This prompted us to study extensively the sequential time-related effects of MTX on cytotoxicity and on purine and pyrimidine metabolism, whereas most studies in the literature only address the effects of MTX at limited points of time. In the present study we are measuring all parameters at those points of time, which proved to be important with regard to the time-related inhibitory effects of MTX on purine *de novo* synthesis [3]. Our study indicates that these time- and dose-related effects of MTX are important for further elucidating the complex mode of action of MTX.

As shown in Fig. 1a, 0.02 μM MTX inhibits cell growth after 20 hr of incubation. At the same time an increase of the fraction of cells in early S phase is noted (Fig. 2a). Although cell viability decreased, clonal growth (Table 2) was not inhibited at 24 hr, indicating that accumulation of cells in early S phase was reversible, when the low concentration of MTX was washed out before the cells were plated on soft agar.

Our studies of the effects of 0.02 μM MTX on intracellular incorporation of radiolabeled hypoxanthine in a concentration of 10 μM [3], showed a marked increase of incorporation at 24 hr of incubation. Thus, addition of fresh medium containing 3–5 μM MTX hypoxanthine could contribute to the recovery of clonal growth due to a recovery from the effects of the partial inhibition of purine *de novo* synthesis [12, 19]. At 48 hr, colony forming activity was reduced to 43.8%, indicating that MTX-cytotoxicity was obvious at that point of time and that the accumulation of cells in the S phase did not recover by addition of fresh medium.

The effect of 0.02 μM MTX on the inhibition of thymidylate synthetase is shown in an initial small decrease of dTTP levels (Fig. 3a). The initial decrease of dGTP and dATP levels between 0 and 20 hr may be caused by consumption, because DNA synthesis is inhibited incompletely, and also by an increasing inhibition of purine *de novo* synthesis. The significant rise of dCTP levels (Fig. 3a) between 0 and 20 hr confirmed our flow cytometry results (Fig. 2a) and earlier findings [5, 13, 14, 18, 28–31] of increased dCTP levels as a phenomenon of accumulation of cells in early S phase. The increased levels may be the result of a diminished inhibition of CDP-reductase by decreased dTTP and dATP levels [27–31]. The further rise of dCTP levels with a maximum at 28 hr is accompanied by a small increase of dTTP, dGTP and dATP between 20 and 28 hr, and may be the result of a decreased consumption for DNA synthesis. Figure 2a demonstrated that most cells were arrested in early S phase between 20 and 28 hr. This arrest is incomplete, because the histogram of DNA distribution at 48 hr indicates a slow progression of a cohort of cells through the S phase. This was accompanied by a decrease of dTTP, dGTP and dCTP levels as occurs in late S phase, indicating an inhibition of all ribonucleotide reductases due to a recovery of dATP levels (Fig. 4a) [27–30].

We noticed significant differences between MOLT-4 cells in our study and CCRF-CEM cells in the studies of others [7, 17–19]. Treatment with

0.02 μM MTX in both T-cell lines resulted in less progression through the S phase of the cell cycle and more pronounced decreases of deoxyribonucleotide levels in CCRF-CEM cells, compared to MOLT-4 cells. This may be due to more pronounced effects of MTX on ribonucleotide pools and RNA synthesis in MOLT-4 cells.

Between 0 and 20 hr of incubation, purine ribonucleotide levels (Fig. 5a) are not significantly impaired, whereas pyrimidine ribonucleotide pools (Fig. 6a) increase. This phenomenon may be due to the partial inhibition of purine *de novo* synthesis with increasing availability of PRPP and concomitant increase of pyrimidine *de novo* synthesis. We did not perform two parameter flow cytometry as described by Taylor and Tattersall [17]. However, our data suggest that RNA synthesis is still ongoing between 0 and 20 hr [9–11], i.e. unbalanced growth. Between 20 and 28 hr a significant increase of all ribonucleotides is noted (Figs 5a and 6a), which coincides with the maximal increase of PRPP levels [3] and which also indicates a decreased utilization of all ribonucleotides. These data suggest an inhibition of RNA synthesis between 20 and 28 hr. The increase of pyrimidine ribonucleotides (Fig. 6a) is more pronounced compared to that of purine ribonucleotides (Fig. 5a). This can be explained by the maximally increased availability of PRPP between 20 and 28 hr, due to partial inhibition of purine *de novo* synthesis, which can be utilized for an increased pyrimidine *de novo* synthesis. The tremendous increase of UMP levels (Fig. 7a) between 20 and 28 hr also indicates an increased pyrimidine *de novo* synthesis and a decreased utilization due to inhibition of RNA synthesis. Thus, the maximal inhibition of purine *de novo* synthesis coincides with the inhibition of RNA synthesis [9–11], i.e. balanced growth (inhibition of both DNA and RNA synthesis). This may explain the differences between MOLT-4 cells and CCRF-CEM cells, because CCRF-CEM cells did not demonstrate balanced growth at 24 and 48 hr [7, 17, 18].

Figures 3a–7a demonstrate that the effects of 0.02 μM MTX almost disappear at 48 hr. However, a further increase of cells in early S phase is noted, associated with a slow progression of a cohort of cells through the S phase. Moreover, dCTP levels remain elevated at 48 hr. These data may seem contradictory. However, an increasing loss of viability (Fig. 1b) is demonstrated at 48 hr and an inhibition of clonal growth (Table 2). The increased cell loss at 48 hr leads to increased nucleic acid breakdown and provides the viable cells with purine and pyrimidine nucleotide precursors. These precursors can be reutilized to produce purine and pyrimidine nucleotides [9–11], consuming PRPP as a cofactor. We demonstrated that PRPP levels at 48 hr decreased to those of untreated cells [3], suggesting PRPP consumption. Moreover, we noted a decrease of AICAR levels at 48 hr, indicating a concomitant recovery of the inhibition of purine *de novo* synthesis.

The decrease of all ribonucleotides at 48 hr (Figs. 5a and 6a), which coincides with the recovery of purine *de novo* synthesis, suggests a consumption of ribonucleotides and a recovery of RNA synthesis in the surviving viable cells, i.e. unbalanced growth. However, the recovery of RNA synthesis did not result in a recovery from cytotoxicity [9–11].

Thus, in MOLT-4 cells, treated with 0.02 μM MTX, the inhibition of purine *de novo* synthesis is accompanied by an inhibition of RNA synthesis and DNA synthesis, i.e. balanced growth, after a period of classical unbalanced growth between 0 and 20 hr, whereas recovery of purine *de novo* synthesis at 48 hr is associated with a recovery of RNA synthesis.

The effects of incubation with 0.2 μM MTX differ in many aspects from those with 0.02 μM MTX, described above. The effect on cytotoxicity and cell cycle distribution (Figs 1 and 2 and Table 2) were described in Results.

The inhibition of thymidylate synthetase is almost complete, leading to a rapid decrease of dTTP levels (Fig. 3b). The complete inhibition of purine *de novo* synthesis [3] is reflected in a rapid decrease of ATP and GTP levels (Fig. 5b). ADP and GDP reductions are impaired due to decreased levels of their activators, GTP, dTTP and ATP [27, 29], leading to decreased levels of dATP and dGTP (Fig. 4b). Because of the initial arrest of cells in G1 phase and a decrease of their activators, the activation of all ribonucleotides is nullified, leading also to decreased levels of dCTP (Fig. 3b). Moreover, the decreased dTTP levels may result in a "de-inhibition" of dCMP-deaminase [7, 28, 29], leading to increased availability of dUMP and a decrease of dCTP. The rapid decrease of all deoxyribonucleotides at 8 hr ultimately prevents cell cycle progression through all parts of the cell cycle (Figs. 2b and 2c).

As mentioned before, the initial decrease of purine ribonucleotides between 0 and 8 hr after incubation with 0.2 μM MTX is a consequence of the complete inhibition of purine *de novo* synthesis. Nevertheless, these effects also suggest a decreasing, but still ongoing RNA synthesis [10, 11] between 0 and 8 hr with consumption of ATP and GTP, i.e. classical unbalanced growth. The initial increases of UMP (Fig. 7b) and UTP and CTP (Fig. 6b) may be the result of an increased pyrimidine *de novo* synthesis due to increased availability of PRPP. They also reflect an increasing inhibition of RNA synthesis [11].

Between 8 and 12 hr after incubation with 0.2 μM MTX, the decrease of ATP and GTP levels ceases, suggesting a complete inhibition of RNA synthesis. This phenomenon also occurred at that point of time, where the inhibition of purine *de novo* synthesis is maximal, as was shown before in cells treated with 0.02 μM MTX at 28 hr. The initial increase of UTP and CTP levels ceases between 8 and 12 hr, and UMP levels do not increase to such an extent as shown in cells treated with 0.02 μM MTX, which indicates that pyrimidine *de novo* synthesis will be diminished.

After 12 hr of incubation with 0.2 μM MTX a significant rise of all ribonucleotides is shown (Figs. 5b and 6b) and the increase of UMP levels continues. These effects are a consequence of nucleic acid breakdown together with a reutilization of nucleotide precursors [10, 11]. Our data from labeled glycine incorporation [3] indicated that the inhibition of purine *de novo* synthesis continues over a period of at least 48 hr after treatment with 0.2 μM MTX. Thus, the consumption of PRPP, due to reutilization

of nucleotide precursors, will ultimately result in a decrease of PRPP levels, as was shown earlier [3]. These events occur at an earlier point of time as compared with MOLT-4 cells treated with 0.02 μM MTX. Moreover, these data suggest a complete inhibition of RNA synthesis and balanced growth after 12 hr of incubation with 0.2 μM MTX. We could not demonstrate a (partial) recovery or stabilization of RNA synthesis, as was shown in our MOLT-4 cells treated with 0.02 μM MTX and was demonstrated also by others [10, 11], using concentrations of MTX up to 1 and 2 μM in other cell lines.

Our data suggest a prominent role of the inhibition of purine *de novo* synthesis with regard to cytotoxicity in MOLT-4 cells having an active purine *de novo* synthesis. Borsa and Whitmore [6] stated that the purineless state tended to prevent efficient cell killing. Our study and those of others [9–11], however, demonstrated a close correlation between the onset of cytotoxicity and maximal inhibition of purine *de novo* synthesis, associated with an inhibition of RNA synthesis (balanced growth).

The role of the inhibition of purine *de novo* synthesis is also demonstrated in the effects of MTX on growth-arrested cells [11] and on normal bone marrow cells and peripheral, non-stimulated blood lymphocytes, which do not have an active purine *de novo* synthesis [4]. Incubation of peripheral blood lymphocytes with 0.02 μM and 0.2 μM MTX did not result in a rise of PRPP levels over a time period of 48 hr (Bökkerink, unpublished data), nor could we demonstrate cytotoxicity. In addition, normal bone marrow cells, treated with MTX [16] did not demonstrate significant changes in deoxyribonucleotide pools. Thus, both the antithymidylate and the antipurine effects of MTX probably contribute to an efficient cytotoxicity in MOLT-4 cells. These phenomena are dose- and time-dependent.

The effects of various concentrations of MTX on purine and pyrimidine nucleotides and on RNA and DNA synthesis are of great importance in order to elucidate the potential synergistic effects of a sequential combination chemotherapy with MTX and 6MP. We demonstrated a close correlation between the maximal inhibition of purine *de novo* synthesis and the maximal intracellular uptake of exogenous labeled hypoxanthine and 6MP [3]. In order to explain the potential synergistic effects after sequential administration of MTX and 6MP, an increased uptake of 6MP into RNA [32] is necessary after pretreatment with MTX, which requires an ongoing RNA synthesis. However, the phenomena presented in this study demonstrated that the inhibition of purine *de novo* synthesis and the increased intracellular uptake of hypoxanthine and 6MP was associated with a decreased RNA synthesis. These phenomena might be explained by a partial rescue of RNA synthesis due to the administration of exogenous hypoxanthine or 6MP.

Therefore, studies are in progress in our laboratory with respect to the incorporation of 6MP, after pretreatment with MTX, into newly formed DNA and RNA, by means of double-labeling techniques with ^{14}C-6MP and ^{32}P.

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