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Methotrexate and its polyglutamate derivatives in erythrocytes during and after weekly low-dose oral methotrexate therapy of children with acute lymphoblastic leukemia

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Summary. Methotrexate and methotrexate polyglutamates were quantitatively determined in red blood cells from 12 children with acute lymphoblastic leukemia who were treated with MTX (15–20 mg/m^2 per week) and daily 6-mercaptopurine orally during the steady-state period of erythrocyte MTX concentration (ery-MTX). The terminal decline of the ery-MTX and its polyglutamate metabolites were determined for up to 15 weeks after cessation of MTX treatment. Methotrexate polyglutamates with 2–4 extra glutamyl derivatives (MTX-glu$_{2-5}$) constituted 75% of the MTX in the entire red blood cell population. MTX-glu$_3$ was the principal metabolite; no MTX-glu$_{6-7}$ was identified. After discontinuation of MTX therapy, the ery-MTX declined in a non-linear manner because of different half-lives for the individual polyglutamates. From about 5 weeks until 13–15 weeks after the last MTX dose, the erythrocyte MTX elimination curve was linear. The approximate half-life of MTX-glu$_1$ was 2–3 days; for MTX-glu$_2$ it was 4–15 days. The concentration of MTX-glu$_{3-5}$ remained constant in the erythrocyte throughout its life span and declined only with age-dependent destruction of the red blood cell. It was calculated that 80%–90% of MTX in newly formed reticulocytes was MTX-glu$_{1+2}$, the remainder being MTX-glu$_{3-5}$. Mature red blood cells did not form methotrexate polyglutamates to any significant degree. There was a significant correlation between the amount of MTX-glu$_{3-5}$ and the steady -state ery-MTX, which to some extent explained the interindividual variation of the ery-MTX in children with ALL in maintenance therapy.

Introduction

Methotrexate (MTX) has constituted the backbone of maintenance therapy of childhood acute lymphoblastic leukemia (ALL) for over three decades. The rate of membrane transport [9, 18], the intracellular drug concentration in excess of the fraction bound to dihydrofolate reductase (DHFR) [8], and the extent of polyglutamylation [5, 7, 19, 24] are responsible for the degree of cytotoxic action of MTX. Drug sensitivity and selectivity have been found to be related to the extent of MTX-polyglutamate metabolism in various tissues [6, 23].

During low-dose weekly therapy, MTX accumulates in erythrocytes [2, 11, 16, 26, 27]. The erythrocyte MTX concentrations (ery-MTX) were correlated to the weekly dose of MTX, although an appreciable interindividual variation was noted [26, 27]. MTX polyglutamates in erythrocytes have been described only sporadically, and no quantitative determinations of the individual polyglutamate forms have been made [2, 10, 15, 20].

MTX concentrations declined with increasing mean cell age in age-fractionated erythrocytes, mainly due to the disappearance of the MTX-mono and MTX-diglutamates (MTX-glu$_1$ and MTX-glu$_2$) [28]. In this study, however, we were not able to decide if polyglutamylation took place in mature erythrocytes, as proposed by some authors [11], or in the erythroid precursors [22].

The aims in the present study were: (1) to describe the quantitative aspects of MTX polyglutamates in erythrocytes in children with ALL; (2) to study the extent to which this was related to the interindividual variation of the erythrocyte MTX concentration; (3) to investigate the pharmacokinetics of the terminal disappearance of MTX and its polyglutamyl derivatives from the erythrocytes. This would provide more insight into the mechanisms responsible for maintenance of the steady-state ery-MTX concentrations during the unchanged weekly therapy of children with ALL.

Material and methods

Patients. Twelve children with ALL, in their first remission, were included in the study. All received maintenance therapy consisting of weekly MTX orally (16.8–21.4 mg/m^2; mean 19.5 mg/m^2) and daily 6-mercaptopurine (6-MP: 75 mg/m^2). No other antileukemic drug had been administered during the last 3 months, and the dose of MTX and 6-MP had been unchanged for a minimum of 8 weeks to ensure steady-state ery-MTX [11, 26]. The ery-MTX was studied in 6 of the children at various times for up to 15 weeks after completion of maintenance therapy; in 3 of these, the polyglutamate distribution was investigated.

MTX-polyglutamate analysis. In order to investigate the MTX polyglutamate distribution, 1–2 ml of red blood cells was hemolyzed in 5 vol of a strong β-mercaptoethanol buffer to inhibit the degradation of MTX-polygluta-

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mates to monoglutamate [17]. After boiling and centrifugation, the sample was further purified on a DE 52 column, eluted with 1 *M* ammonium bicarbonate and lyophilized. The material was reconstituted into 300 µl water, and the MTX polyglutamate separation was carried out by injecting 5–30 pmol of MTX onto a Waters µm bondapack C18 column (part 086684) for high-performance liquid chromatography (HPLC), as described previously [28]. The flow rate was 1 ml/min, and 300 µl fractions was collected every 18 s. MTX concentrations in the individual fractions were determined by the radio-ligand binding assay. Column recoveries were 75%–120% (mean 95%).

MTX-analyses. In the children studied during maintenance therapy, 5 ml EDTA blood was drawn 6–7 days after the last oral dose of MTX. After hemolysis in H_2O, the erythrocyte MTX concentration (ery-MTX) of the boiled supernatant was determined by a radio-ligand binding assay, slightly modified from Kamen et al. [14], using bovine dihydrofolate reductase (DHFR) as binder and ^{3}H-MTX as tracer. The assay had a detection limit of 1 pmol/ml, using 100 µl sample volume and 0.5 pmol/ml when 200 µl was used as sample volume. This corresponded to 0.3 and 0.15 pmol per fraction of HPLC separated MTX polyglutamates. When 7 pmol MTX was injected on the column, 0.15 pmol MTX in a fraction constituted about 2% of the total amount of injected MTX. Thus, when expressed as nmol/mmol Hb, the detection limit of the individual MTX polyglutamates was 0.005 nmol/mmol Hb. In practice, values less than 0.010 were not included. The amount was given in nmol/mmol Hb on the basis of the total erythrocyte MTX concentration and the percentual distribution of the individual MTX-polyglutamates.

Results

Figure 1 shows the ery-MTX in relation to the weekly dose of MTX. A large interindividual variation is noted, and within this limited range of weekly MTX dosage no correlation was observed ($r = 0.11$). Figure 2 shows a representative example of the MTX polyglutamate distribution in the red blood cells from one child during maintenance therapy. The individual peaks are clearly separated, allowing quantitative assessment of the individual polyglutamates. In the entire erythrocyte population, 75% of MTX

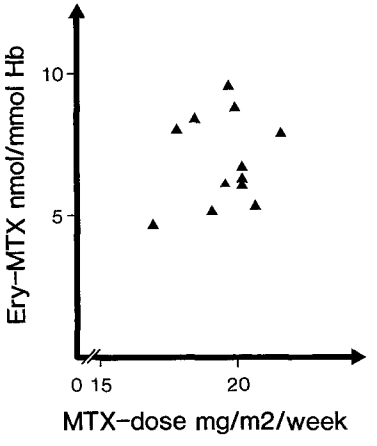


Fig. 1. Steady state ery-MTX (nmol/mmol Hb) in relation to the weekly dose of MTX ($r = 0.11$; P: NS)

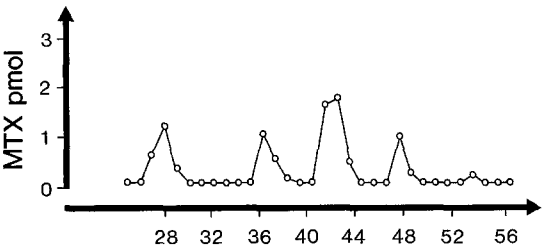


Fig. 2. Representative MTX polyglutamate distribution in erythrocytes from one child. The individual polyglutamates eluted in the following fractions: MTX-glu_1 in fraction 27–29; MTX-glu_2 in fraction 36–38; MTX-glu_3 in fraction 41–43; MTX-glu_4 in fraction 48–49 and MTX-glu_5 in fraction 53

had been metabolized into polyglutamates with 1–4 extra glutamate molecules (MTX-glu_{2-5}). MTX-glu_3 was the predominant metabolite and MTX-glu_5 was detectable in 9 of 12 children, whereas no MTX-glu_{6-7} could be demonstrated (Table 1).

There was a correlation between the steady-state ery-MTX concentration and the amount of MTX-glu_{3-5} in the erythrocytes (Fig. 3). For unknown reasons, one patient showed a different MTX-polyglutamate profile compared with the rest of the group. When this patient was excluded, linear regression analysis showed a significant correlation between the ery-MTX and the amount of MTX-glu_{3-5} ($r = 0.82$, $P < 0.01$).

Table 1. Percentual distribution of the individual MTX polyglutamates (MTX-glu_{1-5}) ± 1 SD in red blood cells from 12 children

MTX-glu_1	25 ± 9.0
MTX-glu_2	20 ± 7.0
MTX-glu_3	37 ± 7.6
MTX-glu_4	15 ± 7.7
MTX-glu_5	3 ± 2.4[a]

[a] MTX-glu_5 was not detected in the erythrocytes from 3 of 12 children

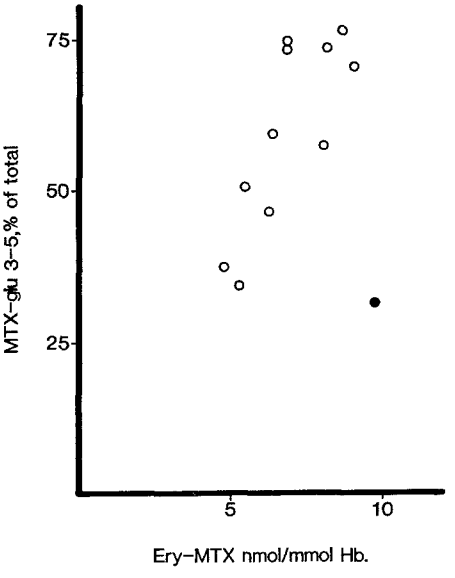


Fig. 3. Amount of MTX-glu_{3-5} (percentage of total amount of MTX) in relation to the steady-state ery-MTX ($r = 0.82$; $P < 0.01$). ●, Excluded from the calculations

In 6 of the children, maintenance therapy was discontinued after 3 years of complete remission, and the ery-MTX was followed for up to 15 weeks after the last MTX dose. Figure 4 shows an initial rapid decline of the ery-MTX, followed by a slower terminal decline which, from about 5 weeks after the last dose of MTX, seemed to be approximately linear.

In 3 children, the MTX polyglutamate distribution in the erythrocytes was investigated in the weeks following discontinuation of MTX therapy (Figs. 5a–c). It was observed that virtually all MTX-glu_1 had disappeared from the erythrocytes 3 weeks after the last dose of MTX. MTX-glu_2 declined less steeply; 7 weeks after discontinuation of MTX this metabolite was no longer detectable. There was no obvious difference in the rate of disappearance of MTX-glu_{3-5} from the erythrocytes during the entire period of observation. The disappearance of these metabolites could be ascribed to the amount lost by destruction of senescent red blood cells. Therefore, we concluded that the concentration of MTX-glu_{3-5} remained constant in the individual red blood cell during its entire life span.

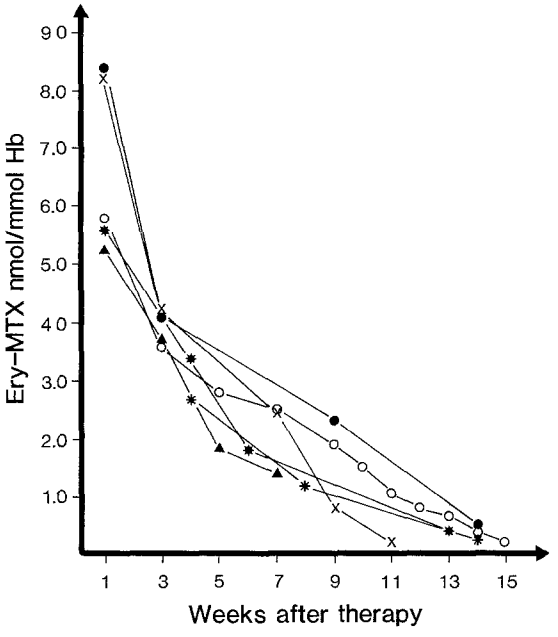


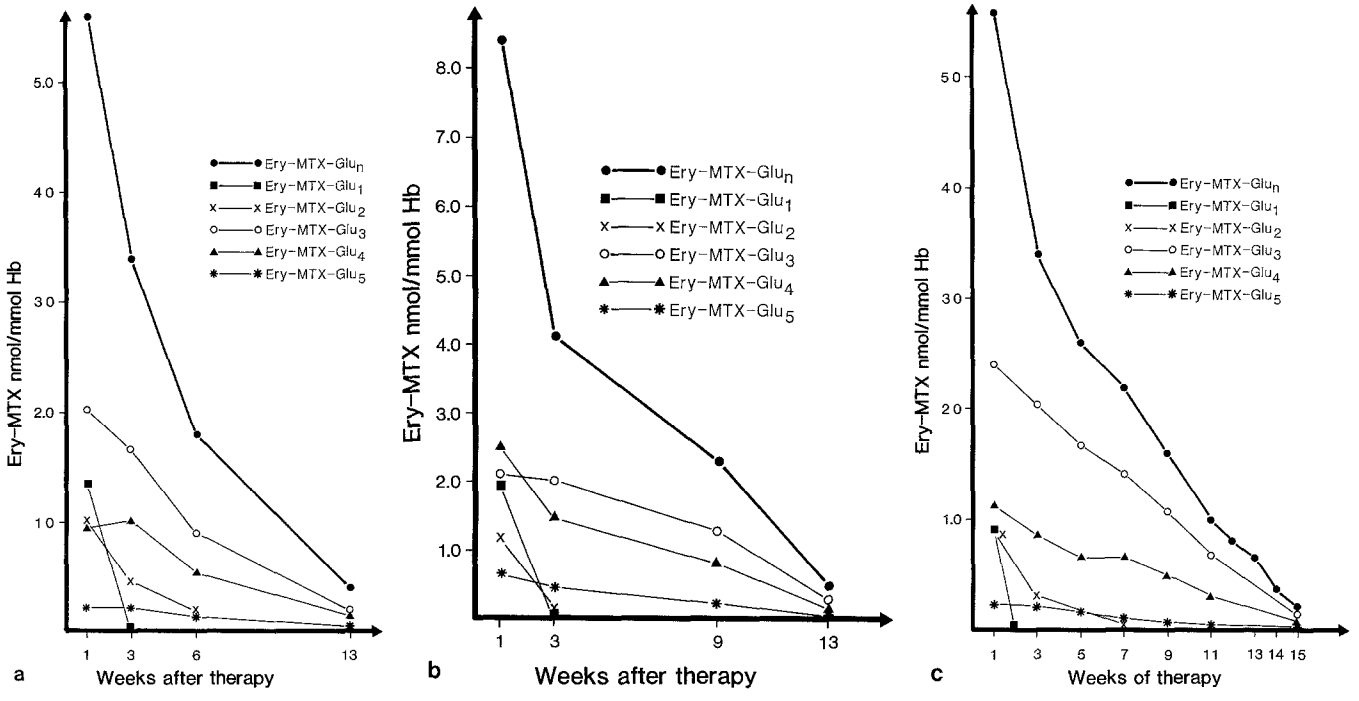
Fig. 4. Disappearance curves of ery-MTX in 6 children after discontinuation of MTX treatment

Discussion

Methotrexate and its polyglutamate derivatives were first detected in human erythrocytes by Baugh et al. [2]. Since then, there have been a few clinical reports on MTX in erythrocytes as a possible mechanism of toxicity to the drug [12, 15, 29].

MTX is metabolized to polyglutamate forms with up to six extra glutamate molecules. The MTX polyglutamates are retained intracellularly for considerably longer periods of time than the monoglutamate forms [1, 13]. This accounts for their increased cytotoxic activity in both neoplastic and normal tissue. The methotrexate polyglutamate concentration in red blood cells may therefore reflect the capacity of normal red blood cell precursors for polyglutamate formation and may thus indicate the impact of MTX on normal hematopoietic bone marrow cells. Besides, the extent of polyglutamylation may be of importance for the long retention of MTX in red blood cells, which has been observed after cessation of maintenance therapy [21, 25].

This report describes, for the first time, the quantitative MTX polyglutamate distribution in red blood cells from



Figs. 5a–c. Disappearance curves of ery-MTX (ery-MTX-glu_n) and the individual MTX polyglutamates from the erythrocytes during the weeks following cessation of MTX therapy in 3 children

children with ALL during low-dose oral MTX therapy on a weekly basis. Kamen et al. [15] demonstrated that MTX polyglutamates accounted for over 60% of MTX in RBCs, but the separation procedure applied did not allow exact quantitation of the individual polyglutamate forms. In our study, 75% of the MTX in the entire population of erythrocytes was polyglutamate derivatives with up to four extra glutamyl recidues (MTX-glu_{2-5}), MTX-glu_3 being the principal metabolite. The red cell precursors in these children were not able to form MTX metabolites with more than five glutamate residues.

The observed correlation between the ery-MTX concentration and the amount of MTX-glu_{3-5} was probably due to the fact that these metabolites remained in the individual erythrocyte during its entire life span, as reported by Krakower and Kamen [21], and thus made a significant contribution to the total ery-MTX concentration. This correlation showed the relative importance of the degree of MTX polyglutamylation for the interindividual variation of the ery-MTX concentration, which was noted both in this report and in a previous one [26].

The MTX polyglutamate distribution in the erythrocytes does not directly reflect the amount of polyglutamates formed by the red cell precursors in the bone marrow. The MTX-glu_1 and -glu_2 disappeared from the red blood cells at a much faster rate than the metabolites with higher glutamate numbers. This was in accordance with previous in vivo results showing preferential disappearance of MTX-glu_{1+2} from age-fractionated red blood cells [28]. From the disappearance curves of the individual MTX polyglutamates, it was unlikely that the MTX-glu_{1+2} had been metabolized into forms with higher glutamate numbers. This mechanism has been proposed to take place in an effort to explain the kinetics of erythrocyte MTX [11], although another study could not demonstrate polyglutamylation in mature erythrocytes from rats [22].

The present study did not allow exact determinations of the half-life of the individual MTX polyglutamates in the erythrocytes, especially for MTX-glu_1 and -glu_2, because blood samples were not frequently taken, during the initial days after discontinuation of therapy. However, from the data available, some approximate half-lives might be indicated: ery-MTX-glu_1, 2–3 days; ery-MTX-glu_2, 4–14 days; ery-MTX-glu_{3-5}, about 60 days, which corresponds to the half-life of the erythrocytes. These figures are in accordance with those reported by Schalhorn et al. [25] after high-dose MTX infusions.

MTX in erythrocytes is probably bound to hemoglobin [3, 4]. Although no binding studies were performed, the disappearance of MTX-glu_1 and -glu_2 observed was thought to be caused by weaker binding of these metabolites to hemoglobin as opposed to that of MTX-glu_{3-5}, among which, however, no differences in rate of disappearance from the erythrocytes could be demonstrated. Benesh et al. [4] demonstrated in vitro that the strength of binding (especially to deoxyhemoglobin) increased with increasing glutamate number. However, no in vivo binding capacities were described, and the binding of metabolites with less than four glutamyl recidues was not investigated.

From about 5 weeks (i.e., after MTX-glu_{1+2} had disappeared), the decline of ery-MTX was almost linear. Extrapolating the linear part of the decline of ery-MTX to week 1 would give the concentration of MTX-glu_{3-5}/mmol Hb at this time. These concentrations were in accordance with the measured concentrations of MTX-glu_{3-5} at week 1 (Table 2). This strongly indicated that the binding of MTX-glu_{3-5}, probably to hemoglobin, was so strong that these metabolites remained in the erythrocytes in largely unchanged concentrations during the entire erythrocyte life span. Thus the terminal decline of the ery-MTX was caused by the destruction of senescent MTX-containing red blood cells after an approximately normal life span.

We have previously claimed that the MTX concentration in a 100% pure reticulocyte population from children with ALL in maintenance treatment with MTX and 6-MP was about 22.4 nmol/mmol Hb [28]. The present results showed that 2.5–4.9 nmol/mmol Hb was in the form of MTX-glu_{3-5}. Since this concentration remained constant throughout the erythrocyte life span, it might be calculated that MTX-glu_{3-5} would account for 10%–20% of the ery-MTX in the reticulocytes that have just emerged from the bone marrow, the remainder (80%–90%) being MTX-glu_{1+2}. This limited polyglutamylation is probably the pharmacological explanation of the relative tolerance of the erythroid cells to MTX in the doses used in maintenance therapy of childhood ALL, since selective action of the drug has been ascribed to the extent of polyglutamate formation in the tissues in question [6].

In conclusion, we have presented quantitative data on MTX polyglutamylation in red blood cells during low-dose weekly MTX treatment, and we have showed that this was related to the overall erythrocyte MTX concentration. MTX with a low number of glutamate residues was rapidly lost from the red blood cells, whereas the metabolites with 3–5 glutamates were retained during the entire erythrocyte life span. These observations serve to shed further light on some of the pharmacokinetic properties of intraerythrocytic methotrexate, which are important when the clinical applicability of measurements of MTX concentration in red blood cells is evaluated.

Table 2. Ery-MTX-glu_{3-5} concentrations at week 1 in three children. Comparison of the measured concentrations and the values obtained from extrapolating the terminal decline of the ery-MTX to week 1 (see text)

Patient	Actual MTX concentration (nmol/mmol Hb)	Extrapolated MTX concentration (nmol/mmol Hb)
1	5.3	4.9
2	3.7	3.7
3	3.2	2.5

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