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Methotrexate pharmacokinetics in age-fractionated erythrocytes*

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Summary. Age fractionation of erythrocytes is useful for further studies of the pharmacokinetics of methotrexate (MTX) in red blood cells. We separated erythrocytes from five blood donors and four patients at different time points after MTX infusions, using discontinuous Percoll gradients consisting of four solutions with a difference of 3% in density among them. The procedure yielded five distinct fractions of erythrocytes of increasing mean cell age as judged by declining reticulocyte enrichment and erythrocyte aspartate aminotransferase activity among the five fractions. MTX concentrations of the erythrocytes were measured at different times in connection with five 24-h MTX infusions (0.7–4 g/m^2) on 14 occasions. Two days after completion of MTX infusion, no MTX was detected in the youngest erythrocyte population in two patients. Seven days after the infusion, the highest MTX concentrations were found in the youngest red blood cells. Ten to fourteen days following the MTX treatment, considerably lower MTX concentrations were found in the young red blood cells, and the MTX-containing erythrocytes seemed to have moved down the gradient. Just before the next MTX infusion (after 28 days) no MTX could be detected in the young erythrocytes. The MTX concentrations at that time were highest in the oldest erythrocyte fractions. This study shows more directly that MTX is incorporated in the red cell precursors of the bone marrow. The pharmacokinetics demonstrated correspond to a maturation time of the erythroblasts of about 7 days.

Introduction

Methotrexate (MTX) a folic acid antagonist, is widely employed in the treatment of acute lymphoblastic leukemia (ALL), severe psoriasis, non-Hodgkin's lymphoma, osteogenic sarcoma, and carcinoma of the head and neck [5, 16, 20]. MTX accumulates in erythrocytes in patients treated with the drug [6, 9, 10, 27]. Intracellularly, MTX is converted to metabolically active polyglutamate forms with up to six extra glutamyl residues [12–14].

In 3–6 days after the first dose the drug reappears in the erythrocytes [3, 6, 25], and it reaches a maximum concentration 14 days after an MTX infusion [25]. Thereafter, the erythrocyte MTX concentration (ery-MTX) declines with a $T_{½}$ which is longer for the MTX polyglutamates than for the MTX monoglutamate [24, 26].

Previous investigations of the pharmacokinetics of MTX in erythrocytes have suggested that MTX is incorporated in the red cell precursors of the bone marrow [3, 25, 29]. Recently it has been shown that the reticulocyte portion of erythrocytes from rats made reticulocytic before MTX administration contained 20–40 times more MTX than mature erythrocytes [13]. Demonstration of MTX in erythrocytes of different ages will provide further knowledge of the intraerythrocytic pharmacokinetics of the drug.

Materials and methods

Patients. Erythrocytes from five healthy blood donors were fractionated in order to establish the method. Thereafter, red blood cells from four patients (one with ALL and three with non-Hodgkin's lymphoma) were fractionated in connection with five 24-h MTX infusions (0.7–4 g/m^2) followed by leucovorin rescue (one patient was studied twice). The patients were investigated 2–28 days after the previous MTX infusion. Two of the patients were studied after their first, two after their third, and one after his sixth MTX infusion. The MTX infusions were administered every 4 weeks.

Collection and preparation of blood. A sample of 15 ml EDTA-blood was collected. Leukocytes were removed by filtering the blood through a filter of α-cellulose (Sigma c 8002) and Sigma-cell 50 (S 5504 Sigma) (1 : 1) washed with 10 ml 0.88% NaCl [1]. Before age fractionation the erythrocyte volume fraction was adjusted to about 0.40 with isotonic NaCl.

Reagents and preparation of gradients. A slight modification of the method described by Salvo et al. [22] was used for preparation of the gradients.

Reagents

Hepes stock 2.66 *M* NaCl, 0.09 *M* KCl, and 0.2 *M* Hepes (*N*-2 hydroxyethylpiperazine-*N*-2-ethane sulfonic acid) pH 7.4;

BSA-H_2O 3.7% 7.4 g bovine serum albumine (Sigma A 8022) was dissolved in 200 ml H_2O and stored at −20 °C in suitable aliquots;

BSA-Percoll 3.7% 7.4 g BSA was dissolved in 200 ml Per-

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coll (Pharmacia Fine Chemicals) and stored at −20 °C in suitable aliquots until use;
Solution 1 19 g BSA-H_2O plus 1 g Hepes stock, pH 6.0;
Solution 2 19 g BSA-Percoll plus 1 g Hepes stock, pH 7.2 (100% Percoll).

Discontinous four-step gradients containing final Percoll concentrations of 60%, 63%, 66%, and 69% were prepared by mixing suitable amounts (by weight) of solutions 1 and 2. Final pH: 6.9, density 1.084–1.095, π: 295–305 mosmol per kg.

Of each of the four Percoll solutions of increasing density 3 ml was carefully poured manually into each of three 15-ml glass tubes so that the solutions did not mix. Finally 1.5–2 ml of the leukocyte-free blood was carefully layered on top of the gradients. The tubes were centrifuged at 1000 *g* for 10 min in a balanced Sigma 3E centrifuge at room temperature.

After centrifugation the erythrocytes from the five bands together with the erythrocytes in the interfaces between the respective bands were harvested manually and washed free of Percoll three times with ice-cold 0.88% NaCl. Before the last washing 10 ml NaCl was added to each fraction and 0.2–0.5 ml was removed for determination of the hemoglobin concentration, from which the percentages of harvested erythrocytes in each of the five fractions were calculated.

Test of age fractionation. This was based on (1) visually observed erythrocyte bands, (2) reticulocyte counts, and (3) erythrocyte-aspartate-aminotransferase activity (ery-ASAT) of the individual fractions. For the *reticulocyte counts,* erythrocytes were suspended in their own plasma before staining with new methylene blue. Of these, 2000 cells were counted, and the results given in percentage of the total erythrocyte counts. For *Ery-ASAT,* erythrocytes were hemolyzed in 40 volumes of a NADP-EDTA-mercaptoethanol-containing buffer (ASAT buffer) [2]. The hemolysates could be stored at −20 °C for 4 weeks without any decline in the ASAT activity. They were centrifuged before assay to remove membrane debris. The ery-ASAT activity was measured by a slight modification of the recommended Scandinavian methods for determination of ASAT [18], and expressed as units per mmol hemoglobin (U/mmol Hb).

Erythrocyte methotrexate analysis. The erythrocytes were hemolyzed in four volumes of the ASAT buffer. When fractions contained less than 200 µl red blood cells, 1000 µl buffer was added to the erythrocytes of these fractions. This volume allowed determination of Hb, ery-ASAT, and ery-MTX. After storage at −20 °C for up to 2 weeks, the samples for MTX determination were thawed, boiled for 7 min, and centrifuged at 9000 *g* for 15 min.

The MTX concentrations of the clear supernatants were determined by a sequential radioligand binding assay, using bovine dihydrofolate reductase (EC 1.5.1.2) as binder and [^{3}H]-MTX as tracer [8]. The assay had a sensitivity of 1 nmol/l and a day-to-day coefficient of variation of 10% at 2 nmol/l (to be published). All MTX concentrations were expressed as nmol/mmol Hb. When multiplied by 20 the MTX concentration may be expressed as nmol/l erythrocytes.

Results

Erythrocyte age fractionation

The Percoll gradient centrifugation produced five separated bands of erythrocytes with a relatively small proportion of cells dispersed in the interfaces between them, which made them easy to harvest. Based on the hemoglobin contents the percentages of cells in the individual fractions were rather constant in the same patient when reexamined on several occasions (data not shown), but a great inter-individual variation was observed (Table 1).

In the upper fraction the ASAT activity was 2.5–3 times higher than in nonfractionated red blood cells, and the reticulocyte counts were 15–30 times higher. Table 1 shows the decline of the ery-ASAT activity and the reticulocyte counts of the individual fractions in relation to the findings of nonfractionated red blood cells. It is seen that these age-related parameters declined between each fraction in both donors and patients. The ratios between fraction 1 and 5 for the ery-ASAT and the reticulocyte counts were 3 : 4 and 40 : 60, respectively.

Methotrexate concentrations in age-fractionated erythrocytes

Erythrocytes from four patients were fractionated at the indicated times in connexion with five 24-h MTX infusions. Figures 1–5 show the methotrexate concentrations in the different erythrocyte fractions. The time after MTX administration and the MTX dose are indicated in each figure.

Figure 1 shows a patient who was studied 7, 9, and 14 days after his first MTX infusion. A uniform decline of the

Table 1. Percentile distribution of red blood cells among the five fractions in the donors and patients. ASAT activity and reticulocyte counts in the five fractions in relation to unfractionated red blood cells (fraction 0). Mean ± SD

Fraction	% erythrocytes		ASAT-activity (fractions 1–5/fraction 0)		Reticulocyte counts (fractions 1–5/fraction 0)	
	Donors (5)	Patients (12)	Donors (5)	Patients (12)	Donors (5)	Patients (8)
1	1.1 ± 0.7	0.6 ± 0.2	2.75 ± 0.44	2.92 ± 0.33	13.8 ± 3.6	27.5 ± 5.1
2	6.2 ± 5.4	2.0 ± 0.8	1.81 ± 0.30	1.96 ± 0.29	5.2 ± 2.8	7.8 ± 3.5
3	22.0 ± 14.8	8.1 ± 3.1	1.35 ± 0.16	1.39 ± 0.17	1.5 ± 0.6	2.8 ± 1.4
4	31.1 ± 12.4	24.5 ± 6.7	1.01 ± 0.14	1.07 ± 0.11	0.7 ± 0.3	1.1 ± 0.8
5	34.9 ± 21.8	64.2 ± 10.2	0.76 ± 0.07	0.91 ± 0.07	0.3 ± 0.1	0.4 ± 0.2
1/5			3.62	3.21	46	60

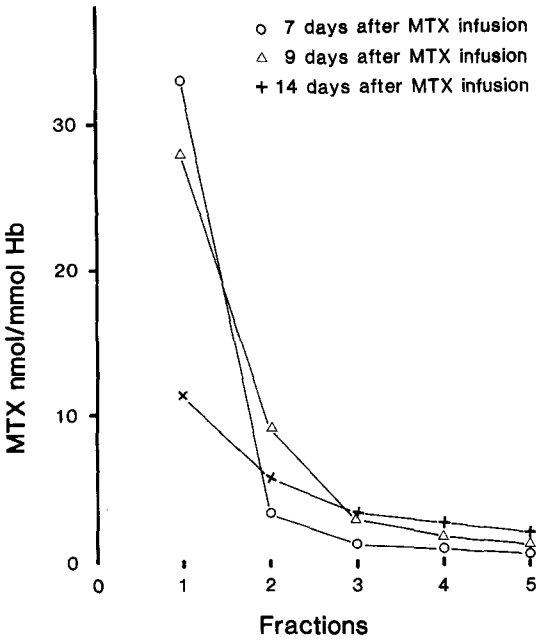

Fig. 1. Methotrexate concentrations in age-fractionated erythrocytes 7, 9, and 14 days after the first MTX infusion (650 mg/m^2)

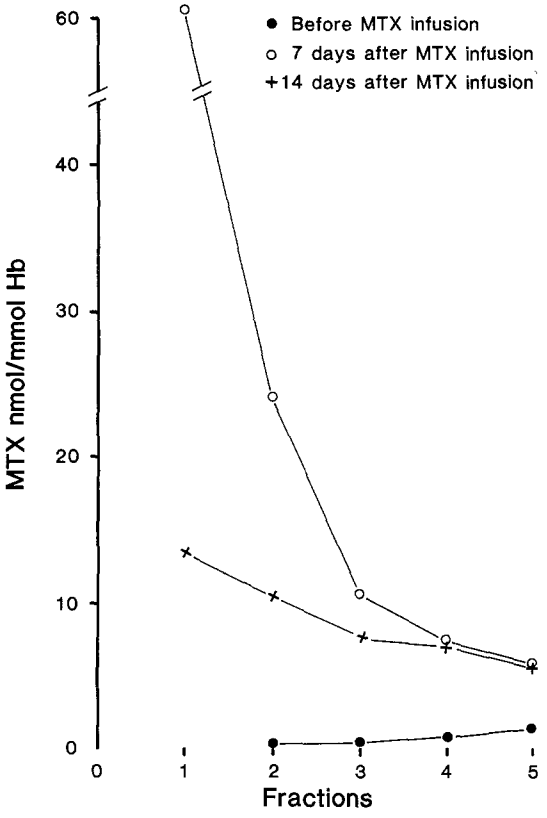

Fig. 2. Methotrexate concentrations in age-fractionated erythrocytes in the same patient as in Fig. 1 after his third 650 mg/m^2 MTX infusion

MTX concentrations between adjacent erythrocyte fractions of increasing mean cell age is demonstrated. The highest concentration is seen in the youngest erythrocytes (fraction 1) on day 7. The ratio of MTX concentrations between fraction 1 and 5 declined with time after the MTX

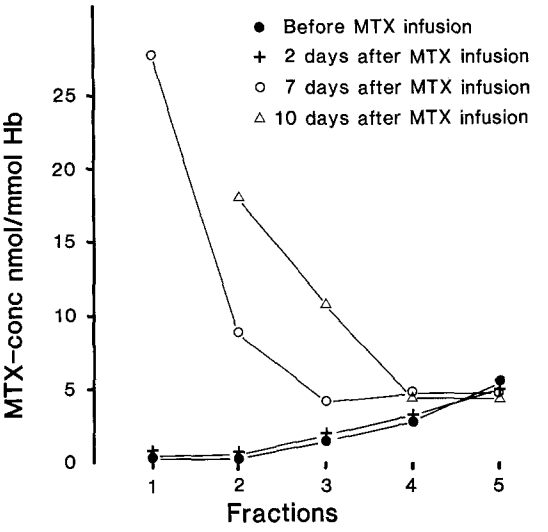

Fig. 3. Methotrexate concentrations in age-fractionated erythrocytes after the third MTX infusion (750 mg/m^2)

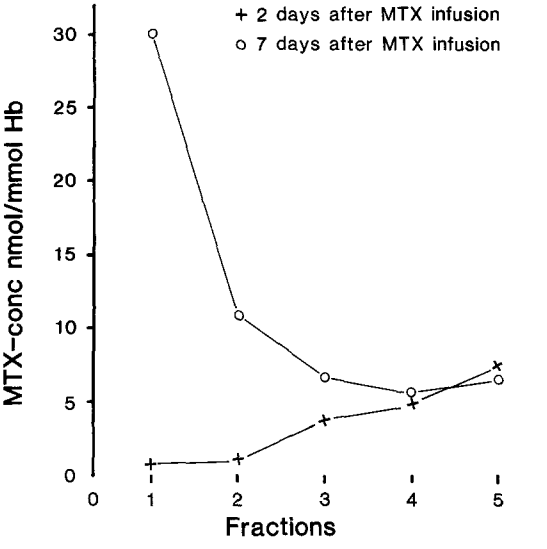

Fig. 4. Methotrexate concentrations after the sixth MTX infusion (4 g/m^2)

infusion and was calculated at 66, 35, and 6 on days, 7, 9, and 14, respectively. Figures 2–4 show the ery-MTX in three patients before (= 0) and up to 14 days after the third (two patients) and the sixth MTX infusion. Before the infusion no MTX was detectable in the youngest erythrocyte fraction, but the ery-MTX increased with increasing mean cell age (Figs. 2 and 3). Two days after the completion of the MTX infusion (serum MTX concentration <25 nmol/l) MTX was still not found in the youngest fractions (Figs. 3 and 4). After the four infusions, in which the renal excretion of MTX was normal, the peak concentration occurred in the young red blood cells on day 7 (Figs. 1–4). Thereafter the MTX concentrations declined in fraction 1, increased in fractions 2 and 3 and remained more or less unchanged in the two oldest fractions (fractions 4 and 5) (Figs. 1–3). Figure 5 shows the results from a patient with a markedly delayed renal MTX excretion (se-MTX 2800 nmol/l 48 h after the infusion) after his first

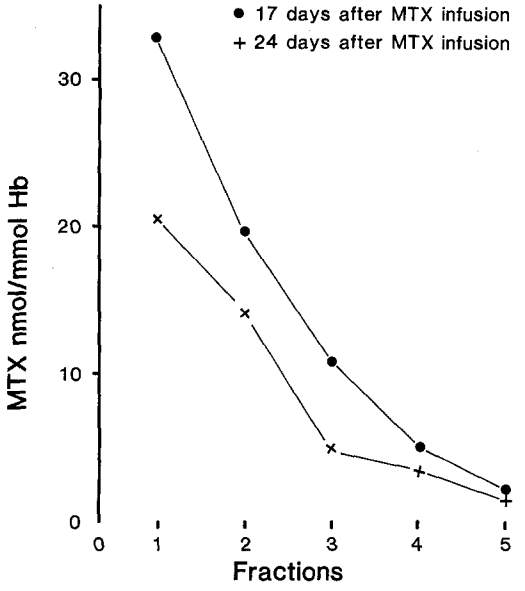

Fig. 5. Methotrexate concentrations 17 and 24 days after the first MTX infusion (1000 mg/m^2) complicated by delayed renal MTX excretion.

MTX infusion. On day 17 (se-MTX <2 nmol/l) a uniform decline of the ery-MTX between the fractions was still observed, and the ratio of the ery-MTX between fractions 1 and 5 was 16. This fraction did not change substantially after another week.

Discussion

Age fractionation of erythrocytes is based on the observation that red cell density increases with increasing cell age [4, 17]. The activity of a number of intraerythrocytic enzymes declines with increasing red blood cell age [15, 19, 22, 23, 28]. Sass [23] found that ery-ASAT was a sensitive parameter of age differentiation. Recently ery-ASAT activity was found to be the most sensitive parameter of red blood cell age among a number of intraerythrocytic enzymes using the same Percoll fractionation procedure as is described in this report [15].

The reticulocyte enrichment and the relative decline of ery-ASAT activity among the five fractions depended on the percentage of red cells in each fraction, which has also been shown by others [15, 28]. The distribution of erythrocytes among the fractions showed a small intrapatient variation, whereas a significant interpatient variation was observed, which was also reported by Salvo et al. [22].

To study the MTX kinetics in newly formed erythrocytes, the applied four-density Percoll gradients yielded a fast and satisfactory age fractionation, especially of the younger red blood cell population as judged by ery-ASAT activity and reticulocyte counts.

On the basis of pharmacokinetic studies of MTX in nonfractionated erythrocytes, others have suggested that MTX is incorporated in the red cell precursors of the bone marrow before appearing in the erythrocytes of the circulation [3, 25, 29]. In the present study the high MTX concentrations on day 7 in the youngest erythrocytes provide more direct evidence for this hypothesis. Recently, high concentrations of MTX were demonstrated in the reticulocytes of rats in which erythropoiesis had been stimulated before MTX administration. The reticulocytes were found to contain 20–40 times more MTX than the mature erythrocytes 4 days after drug administration [14]. The present study showed that 7, and 9 days after the first MTX infusion the reticulocyte-rich fraction 1, contained 66 and 35 times more MTX than the oldest cells of fraction 5 (Fig. 1). Similar figures may be calculated in the three patients studied after their third and sixth infusions if the ery-MTX concentrations in fraction 5 of day 0 or 2 is subtracted from the value of day 7. This again shows that this method successfully retains the young erythrocytes in the upper fractions.

After day 7 progressively lower MTX concentrations were found in fraction 1 up to day 28, when MTX was no longer measurable in the newly produced erythrocytes. The flattening of the curve and the fact that the curves for days 9, 10, and 14 crossed the curve for day 7 indicated that the MTX-loaded, newly formed red blood cells had moved down through the gradients as their age had increased (Figs. 1–3).

The observed MTX concentration peak of fraction 1 on day 7 and the progressive decrease of concentration in this fraction from day 9 to day 28 indicated that the erythroblasts maturing during that period contained increasingly less MTX. These observations are consistent with a maturation time of the MTX-containing erythroblasts of about 7 days, which is widely accepted [30].

The patient whose results are shown in Fig. 5 had a markedly delayed renal excretion of MTX and accordingly received vast amounts of leukovorin for 8 days, which successfully prevented the development of toxic symptoms. Large amounts of leukovorin are known to inhibit the metabolism of MTX to intracellular-retainable and metabolically active polyglutamate forms [7, 11, 21]. Although we could not separate MTX polyglutamates, the supposed inhibition of MTX polyglutamate formation might explain why the two MTX curves of Fig. 5 did not cross, as seen in Figs. 2–4, since MTX monoglutamate has a much shorter intraerythrocytic half-life than the polyglutamate derivatives [24, 26]. If the renal MTX excretion had been normal, the MTX content of the youngest erythrocytes of fractions 1 and 2 would probably have been near to zero on day 24, as seen in Figs. 2 and 3 where no MTX was detected in these fractions 28 days after the last MTX infusion (just before the next infusion).

During the separation some MTX effluxed from the erythrocytes, resulting in a variable recovery. Relatively more of the MTX in the newly formed erythrocytes will be MTX polyglutamates with low numbers of glutamyl residues, which are known to efflux from the cells at a faster rate than MTX with higher numbers of glutamate molecules [26]. Therefore if the efflux of MTX could be reduced the differences of the MTX concentrations between the fractions might be even more pronounced. Preliminary results from our laboratory have shown that fractionation in Percoll gradients cooled to 4 °C will improve MTX recovery.

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