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Methotrexate kinetics in myeloid bone marrow cells and peripheral neutrophils*

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Summary. The pharmacokinetics of methotrexate (MTX) in the proliferating and the maturing myeloid compartments of the bone marrow and in the neutrophils of the peripheral blood was investigated in four patients with malignant non-Hodgkin's lymphoma after treatment with 24-h MTX infusions (500–790 mg/m^2) followed by leukovorin rescue.

1. The myeloid bone marrow cells were separated into two fractions, using a two-step discontinuous Percoll gradient with densities of 1.076 and 1.095 g/ml. The upper fraction consisted predominantly of immature bone marrow cells plus lymphocytes, and the lower fraction contained the mature myeloid bone marrow cells.

2. Cells in the proliferating (immature) myeloid compartment took up and retained MTX to a much greater extent than the mature myeloid cells and the neutrophils.

3. Two days after the MTX infusion no MTX was detected in the neutrophils in spite of a general rise in the total neutrophil count.

4. MTX reappeared in the neutrophils on day 8 in concentrations not related to the concentrations in the proliferating myeloid cells during the MTX infusion.

5. The time of reappearance of MTX in the neutrophils was in accordance with the time it takes for cells in the proliferating pool of the bone marrow to mature and be released into the circulation.

6. No neutropenia was seen after the MTX infusions.

Introduction

Methotrexate (MTX) is given in high-dose infusions (0.5–20 g/m^2 for 6–24 h) in the treatment of various neoplastic diseases [3]. This treatment can only be tolerated by the patients if it is followed by folinic acid (citrovorum factor, leucovorin); otherwise severe, or even fatal, myelosuppression may occur [30].

MTX accumulates to various degrees in different tissues [1, 17, 19, 24]. To what extent this observation is relevant to the understanding of drug toxicity in clinical practice still has to be determined. Kinetic studies of MTX in erythrocytes have suggested that the drug is incorporated in the bone marrow [18, 23, 25], although the demonstration of MTX in the erythroblasts is still lacking.

MTX in bone marrow cells accumulated in vivo and the degree of kinetic disturbances of cell division in this compartment have been found to correlate with the serum MTX concentration, but the MTX kinetics of the myeloid cells in this compartment has not been described further [29].

In order to investigate the pharmacokinetic behavior of MTX in myeloid cells more closely a method of separating the immature (i.e., belonging to the proliferating myeloid cell pool) from the mature cells (belonging to the maturating myeloid cell pool of the bone marrow) by using a two-step discontinuous Percoll gradient was used and is described in the present paper.

The purpose of the study was to determine variations in the MTX content of the myeloid cells during, and up to 2 weeks after, MTX infusions and to relate the findings to the concept of bone marrow incorporation and myelotoxicity of MTX.

Material and methods

Four patients with non-Hodgkin's lymphoma without bone marrow involvement were included in the study. Patient characteristics are seen in Table 1.

The treatment was uniform and consisted of monthly CHOP (cyclophosphamide 750 mg/m^2 i.v., hydroxyldaunomycin (Adriamycin) 50 mg/m^2 i.v., Oncovin 1.4 mg/m^2 i.v., and prednisone 100 mg p.o. for 5 days). Two weeks after each CHOP course the MTX infusions were given, one-third over 30 min and the rest as a continuous infusion for 23.5 h using a peristaltic pump. At 6 h after termination of the MTX infusion leukovorin rescue therapy was initiated: 12 mg i.v. every 4 h hours for six doses, and thereafter 6 mg every 6 h until the serum MTX concentration was below 100 nmol/l.

All the patients were examined in relation to their first MTX infusion. Bone marrow samples were taken after 20 h of constant MTX infusion and on days 3 and 8 after initiation of the MTX treatment. For this, 2–3 ml of the marrow blood was collected in 3 ml cold 0.15 *M* NaCl containing 500 units heparin and 200 units Varidase to prevent cell clumping. At the same time, 20 ml heparin

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Table 1. Patients' data

Patient	Sex	Age	Histology	Stage	MTX mg/m^2
A	M	36	Lymphoblastic lymphoma	IVB	570
B	M	38	Malignant histiocytosis	IA	790
C	M	28	Lymphoblastic lymphoma	IVB	500
D	M	29	Malignant histiocytosis	IVB	690

blood was taken for isolation of neutrophils, and this was repeated in two of the patients 11 and 14 days after the MTX treatment.

Two-step discontinuous Percoll gradients (density 1.076 and 1.095) were prepared as described elsewhere [9]. After overlayering of the bone marrow sample and the blood the gradients were centrifuged for 25 min at room temperature at 600 *g*. The immature bone marrow cells were layered on top of the lighter Percoll solution, and the mature bone marrow cells and the neutrophils of the peripheral blood could be isolated from the top of the heavier solution. The cells were harvested and washed three times in 0.15 *M* ice-cold NaCl.

Before the final washing procedure erythrocytes were removed by hemolysis with 3 vol of cold H_2O for 90 s, followed by the addition of 1 vol of cold 0.60 *M* NaCl to restore isotonicity. After the final centrifugation the cells were reconstituted in 600–1000 µl NaCl and the number of cells counted on a Coulter Counter Plus. Before freezing at −20 °C a smear of the cells was made for differential counting. Before analysis the cells were thawed, sonicated for 30 s, boiled for 7 min, and centrifuged at 9000 *g* for 15 min. MTX concentrations were measured in the clear supernatant and expressed as picomoles per 10^9 cells.

The smears were stained with May-Grunwald-Giemsa stain, and 300 cells were counted under the oil immersion lens. In the stained smears fewer than 10% of the cells appeared to be damaged.

MTX concentrations in cells and plasma, when below 100 nmol/l, were determined by a sequential radioligand-binding assay slightly modified from that of Kamen et al. [11]. The assay measures MTX and MTX polyglutamates equally well (B. A. Kamen 1985, personal communication) and had a sensitivity of 1 nmol/l and day-to-day variations for controls 2 and 10 of 13% and 6%, respectively. Serum MTX concentrations during the MTX infusions were measured with an enzymatic assay with a sensitivity of 25 nmol/l.

Ethical aspects. This study was approved of by the local ethical committee, and the patients agreed to participate after thorough written and oral information.

Results

Table 2 shows the distribution of the bone marrow cells in the upper and lower fractions of the Percoll gradient and, for comparison, the distribution of cells in unfractionated bone marrow. Most of the myeloid cells in the upper portion were myeloblasts, promyelocytes, myelocytes, metamyelocytes and lymphocytes, with only about 15% band and segmented neutrophils. In the lower fraction, however, 85% of the cells were either band or segmented neutrophils. Compared with unfractionated bone marrow, the upper fraction contained four times more proliferating bone marrow cells (myeloblasts, promyelocytes, and myelocytes).

Table 2. Differential counts of unfractionated ($n = 4$) and upper and lower fractions of fractionated bone marrow cells ($n = 12$)

	Unfractionated	Upper	Lower
Myeloblasts	0	1.8±0.3	0
Promyelocytes	0.5±0.3	7.4±0.8	0
Myelocytes	7.5±1.8	26.4±1.9	4.2±0.9
Metamyelocytes	9.3±1.3	12.8±1.2	7.7±1.2
Band-formed neutrophils	14.5±2.8	12.0±1.9	35.0±2.2
Segmented neutrophils	15.3±3.6	3.1±0.7	49.1±2.9
Lympho-monocytes	29.3±3.0	27.7±2.8	1.0±0.3
Erythroblasts	23.8±2.5	0	0

Figures represent means ± SEM, expressed in percentages; 300 cells were counted

Table 3. Mean methotrexate concentrations (picomoles/10^9 cells ± SEM) in the three myeloid compartments in the four patients

	Immature	Mature	Neutrophils
Day 1	1315±121	541± 93	218±37
Day 3	786± 97	332±110	20±11
Day 8	315± 30	846±204	667±59
Day 11	ND	ND	396±77[a]
Day 14	ND	ND	105±16[a]

[a] Two patients examined

The steady state serum MTX concentration during the infusions varied between 5100 and 8500 nmol/l (data not shown). Since the serum MTX levels 44 h after termination of the MTX infusions were below 100 nmol/l in all patients, their renal MTX excretion was considered normal.

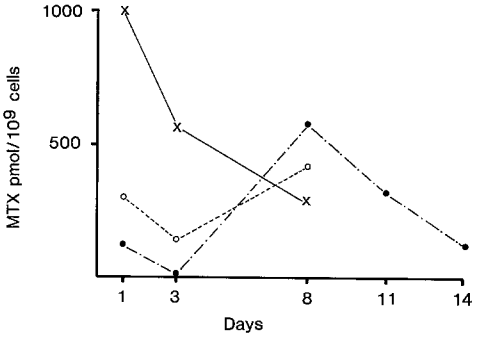

Fig. 1. Patient B: MTX concentrations (pmol/10^9 cells) in the immature bone marrow cells (x———x), the mature bone marrow cells (O------O), and the circulating neutrophils (●—·—·—●)

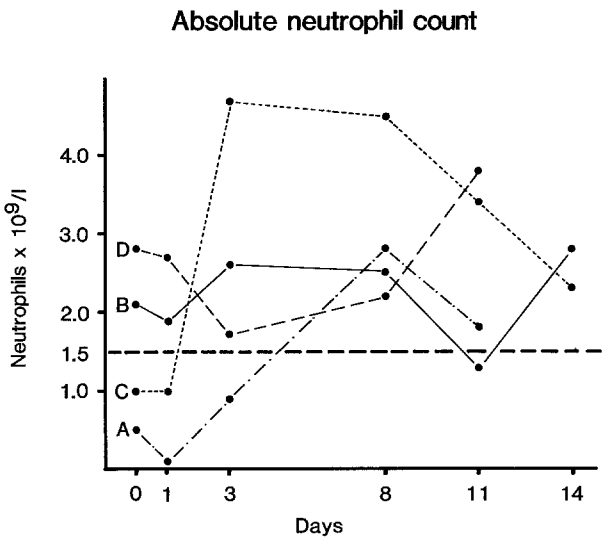

Fig. 2. Total neutrophil counts of the four patients before the MTX infusion (day 0), during the infusion (day 1) and 3–14 days after the start of the infusion

Figure 1 illustrates a typical profile of the MTX concentrations in the immature and mature bone marrow cells, and also in the neutrophils of the peripheral blood of patient B. Among the four patients a quite constant pattern of MTX distribution was seen in the three cell compartments during the investigation period. Table 3 shows the content of MTX in each of the three compartments during and after the infusions. The MTX concentrations were highest in the immature cells both during the infusion and on day 3. There was a positive correlation between the serum MTX during the infusion and the MTX concentration in the immature cells both on day 1 ($r=0.70$) and on day 3 ($r=0.83$). These correlations were not significant, however, probably because of the few patients examined. On day 8 the MTX concentrations in the immature bone marrow compartment had declined to about 25% of the peak value.

During the infusion the mature bone marrow cells contained appreciably less MTX than the immature cells. On day 3 the MTX content of the mature cells was lower, but there was then a substantial increase on day 8. There was no correlation between the serum MTX during the infusion and the MTX content of the mature bone marrow cells.

The MTX kinetics of the neutrophils of the peripheral blood resembled that of the mature cell pool of the bone marrow. During the infusion the MTX concentrations of the circulating neutrophils of all four patients varied between 120 and 278 pmol/10^9 cells. In one patient a slightly higher concentration was observed on day 10, but on day 14 the neutrophil MTX concentration had declined to 20% of the peak value. There was no correlation between the MTX concentrations in the immature cells of the bone marrow on day 1 and the neutrophil MTX concentration on day 8.

To study to what extent MTX incorporation in the myeloid bone marrow cells caused neutropenia the total neutrophil counts of the four patients during the observation period are shown in Fig. 2. Two of the patients had absolute neutropenia ($< 1.5 \times 10$/l) at the start of the infusion, but made a fast recovery. Apart from one transient episode of neutropenia on one patient, on day 11 neutrophil counts were $> 1.5 \times 10^9$/l.

Discussion

MTX acts by arresting cell division in the S phase of mitosis [29] as a result of blocking intracellular enzyme systems, primarily dihydrofolate reductase (DHFR), but also thymidylate synthetase [15]. The degree to which cell division is impaired depends on the MTX concentration in the cells [7, 26]. Therefore, the MTX concentration, especially in the proliferating cell pool of the bone marrow, might be expected to be related to the ability of these cells to continue cell division.

One of the limitations of MTX therapy is its myelosuppressive action. After high-dose infusions myelotoxicity may largely be avoided by the administration of reduced folates (folinic acid), especially in patients with a slow renal MTX excretion [5, 27].

MTX has been shown to accumulate in bone marrow cells in vivo and in vitro, both in humans and in experimental animals [6, 16, 21, 24, 29, 31]. MTX is metabolized to polyglutamates [6, 16, 18, 21, 31], which leave the cells at a slow rate compared with MTX monoglutamate [16]. Vogler et al. [29] demonstrated MTX in bone marrow cells and leukocytes in patients receiving MTX infusions, and demonstrated by flow cytometry an accumulation of bone marrow cells in the mitotic S phase during the MTX infusion. The plasma MTX concentration correlated with the bone marrow cell concentration, but no fractionation of the bone marrow cells or the leukocytes from the peripheral blood was performed.

The present paper demonstrates a method of separating bone marrow cells into mature and immature myeloid cells on the basis of differences in gravity among these cells [20], yielding an eight-fold enrichment of proliferating bone marrow cells in the upper fraction compared with the lower fraction. The hemolysis procedure which was necessary to remove erythrocytes from the myeloid cells also caused hemolysis of the hemoglobin-containing erythroblasts of the bone marrow. This explains why only a few erythroblasts were counted after the fractionation (data not shown).

In the four patients a uniform picture of MTX kinetics in the myeloid cells of the bone marrow and the peripheral blood was demonstrated.

The immature cells of the bone marrow had a greater ability to accumulate and retain MTX, probably as polyglutamates, than the mature myeloid cells and the peripheral neutrophils, both during the infusion and 2 days later, when the serum MTX concentrations were below 100 nmol/l. The MTX concentrations in the proliferating myeloid cells are probably underestimates because of the admixture of about 15% mature neutrophils and about 30% of lymphocytes, which in vitro have been shown to take up even less MTX than the neutrophils [13]. The present demonstration of high MTX concentrations in the proliferating cell pool of the bone marrow is consistent with the findings of Vogler et al., who found a transient inhibition of cell division after 24-h MTX infusions [29].

The difference in the MTX content of the mature cells from day 1 to day 3 may be explained by the efflux of unbound or unmetabolized MTX after termination of the in-

fusion, but might also be caused by resuming of cell division after institution of Leucovorin rescue, since the intracellular MTX content would be halved by each cell division. The difference of MTX in the proliferating cell pool between day 3 and 8 probably reflected this 'dilution effect', caused by the cell divisions in this compartment.

MTX measured in the peripheral neutrophils during the infusion was thought to be a result of simple diffusion, which has also been demonstrated in leukocytes in vitro [13] and in eryhtrocytes in vivo [8]. This fraction of MTX diffuses out of these cells parallel to the plasma MTX decline, probably because neutrophils and erythrocytes [18] are not able to bind MTX or to metabolize it to polyglutamates.

The time interval between the MTX infusion and the reappearance of MTX in the neutrophils of the peripheral blood suggests that MTX is incorporated in the proliferating cells of the bone marrow. On day 3 the neutrophil counts were higher in three of the patients, probably as a result of mobilization of segmented neutrophils from the bone marrow [28]. The fact that no MTX could be detected in the neutrophils in the peripheral blood on day 3 proved that very small amounts of MTX, if any, were retained in the mature myeloid cells of the bone marrow. The MTX measured in this compartment may be explained by the percentage of immature myeloid cells (average 15%) included in this fraction.

The reappearance of MTX in the peripheral neutrophils on day 8 was consistent with the time it takes for cells in the myelocyte pool to mature and be released into the circulation as segmented neutrophils [2, 28]. The wide variation in the maturation time of the myeloid bone marrow cells [14] probably explains the absence of a correlation between the MTX content of the immature cells during the MTX infusion and the MTX content of the circulating neutrophils on day 8. Besides, the degree to which the marginal neutrophil pool contributed to the total circulating neutrophil count was not known. MTX concentrations in reticulocyte-enriched fractions of erythrocytes also reached a peak value 7–8 days after a 24-h MTX infusion, as demonstrated in another study from our laboratory [25].

Because MTX is myelotoxic we studied the implication of the MTX accumulation in the myeloid bone marrow cells for the circulating neutrophil counts of the patients during and after the MTX infusions. Two of the patients had neutropenia at the start of and during the MTX infusion, probably as a result of the previous CHOP treatment. Eight days after the infusion, when the myelosuppressive action of MTX should be at its greatest, the neutrophil counts of all patients exceeded 2×10^9/l. A similar overshoot reaction of the neutrophil count to that observed in two of our patients was also observed in another study of one patient after even larger amounts of MTX [4]. This might be due to the observed normal renal excretion of MTX and the accompanying folinic acid rescue therapy.

Whether the observed amounts of MTX in the proliferating cell pool would induce myelosuppression in the absence of folinic acid is not known. However, a concentration-response relationship could not be claimed on the basis of our studies, since we did not have the opportunity of studying the myeloid cell MTX concentrations in patients ultimately developing myelosuppression after MTX infusions. Such studies would provide further clarification of the basis of MTX's myelosuppressive action.

The formation of MTX polyglutamates in highly purified myeloid precursor cells in vitro has been found to be concentration- and time-dependent [16], and the amounts of intracellular accumulated polyglutamates were correlated to the observed reduction of the formation of colony-forming units [6, 16]. To improve our knowledge of the myelosuppressive action of MTX in clinical material obtained in vivo it would be relevant to investigate how the myeloid cells metabolized MTX to polyglutamates, and how this was affected by the folinic acid rescue therapy, since there is recent evidence that cells with a high folinic acid content seem to have a reduced capacity for accumulation of MTX and for metabolizing MTX to polyglutamate forms, leading to a reduced cytotoxic action of the MTX [10, 12, 22].

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