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Methorexate in neutrophils in children with acute lymphoblastic leukemia

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Summary. Methotrexate (MTX) and 6-mercaptopurine (6-MP) are used for maintenance therapy of acute lymphoblastic leukemia (ALL) of childhood, and both are myelotoxic. Clinically the individual tolerance to the two drugs is variable. In order to study to what extent the MTX accumulation in circulating neutrophils is related to the absolute neutrophil count (ANC), neutrophils were isolated on a discontinuous two-step Percoll gradient in 16 children with ALL in maintenance therapy. The MTX concentration in the neutrophils was determined with a sensitive radioligand binding assay. In all children except one who admitted noncompliance, MTX was found in the neutrophils in concentrations (56–460 pmol/10^9 cells) positively correlated with the weekly dose of MTX ($r=0.51$, $p<0.01$). The interindividual variation was large. Children with relatively low neutrophil MTX exhibited the widest intraindividual variation of neutrophil MTX upon reexamination during continued MTX administration with the same dosage schedule. Increases in the weekly dose of MTX resulted in proportional increases in the neutrophil MTX. In half the cases the ANC was $<1.5\times10^9$/l. The ANC was not related to the weekly MTX dose or the daily 6-MP dose. In children with ANC $>1.5\times10^9$/l there was a significant inverse correlation between the ANC and the neutrophil MTX ($r=-0.71$, $P<0.01$), which was not found in the group of children with ANC $<1.5\times10^9$/l. These findings may be explained by differences in the kinetics of the granulopoiesis between children with high and children with low neutrophil counts.

Introduction

Methotrexate (MTX) is used as weekly peroral therapy in combination with daily 6-mercaptopurine (6-MP) for maintenance treatment of acute lymphoblastic leukemia (ALL) in childhood.

Both drugs are myelosuppressive and during maintenance therapy of ALL a reduction of the total leukocyte count to $2-3\times10^9$/l is intended. However, the individual tolerance to the two drugs is variable, and only partly dependent on the dosage [12]. So far no satisfactory explanation has been found for the pharmacological basis of the myelosuppression seen during the maintenance phase of ALL treatment. A high concentration of 6-thioguanine (a metabolite of 6-MP) in the erythrocytes was found to correlate with the absolute neutrophil count (ANC) especially in neutropenic patients [7]. When MTX was used we found no correlation between the MTX accumulation in the erythrocytes and signs of myelosuppression in 53 children in maintenance treatment for ALL [14].

The neutrophils are present in the circulation for less than 24 h [4]. We felt the MTX content of these cells might be more directly related to the ANC, since MTX seems to be incorporated into, and exert its myelosuppressive effect in, the proliferating myeloid cells of the bone marrow [13, 16].

The purpose of this paper was to describe MTX concentrations in the neutrophils and relate this finding to the dose of MTX and the degree of myelosuppression judged on the basis of the ANC in children with ALL in maintenance treatment.

Materials and methods

Patients. All children with ALL in maintenance treatment in our pediatrics department during the period of investigation were included in the study. There were 16 children in the study group; all were in their first remission. The induction treatment depended on the risk classification of the individual patient. Maintenance therapy consisted of MTX 20 mg/m^2 weekly p. o., taken as 2.5-mg tablets (Lederle), and 6-MP 75 mg/m^2 daily. No additional chemotherapy had been given for at least 3 months prior to the investigation. At the time of the investigation the MTX and 6-MP doses had been unchanged for at least 3 weeks. We performed 27 neutrophil separations in the 16 children. We reexamined 2 patients after alteration of the weekly dose of MTX and 8 after 1–2 months of unaltered MTX medication.

All children were seen in the outpatient clinic 6–7 days after the previous MTX dose, and 20 ml blood was taken from each into tubes with heparin (Heparin Lithium, Venoject VT 100 HL) for isolation of neutrophils. Further blood samples were taken into tubes with EDTA for a complete hematologic status performed on a Coulter Counter Plus. When the leukocyte count was below 2.5×10^9/l the number of leukocytes was counted manually using a Bürker-Türk chamber. A peripheral blood smear was stained with May-Grünwald-Giemsa for the differential count; 100 leukocytes were counted.

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Separation of neutrophils. This was accomplished by a method developed in our laboratory, which has been described elsewhere [8]. A two-step discontinuous Percoll gradient (densities: 1.076 and 1.095) was used, and the leukocytes were separated in two distinct bands. The upper band consisting of the mononuclear cells was discarded. The lower bands consisting of the neutrophils were harvested and washed three times with ice-cold 0.15 *M* NaCl. Erythrocytes were removed by hemolysis with 3 vol H_2O for 90 s, after which isotonicity was restored with 1 vol 0.60 *M* NaCl. The neutrophils were suspended in 600–700 µl NaCl, and the leukocyte count was performed as a microtest on a Coulter Counter plus. The average yield of neutrophils was 73% after the hemolysis procedure. The supensions of neutrophils were >99% pure.

Methotrexate analysis. The harvested cells were frozen at −20 °C for less than 4 weeks, then thawed, sonicated for 30 s, boiled for 7 min, and centrifuged at 9000 *g* for 15 min. MTX concentrations were determined in the clear supernatant by a modification of a sequential protein-binding assay with a range of 0–16 nmol/l described by Kamen et al. [9]. Controls with 2 and 10 nmol/l varied by 13% and 6%, respectively, from day to day; sensitivity was 1 nmol/l. MTX concentrations below 1 nmol/l in the supernatants were disregarded.

The mean MTX concentration was 12.2 nmol/l (range 1.1–34.5 nmol/l). On the basis of the neutrophil counts in the supernatants, the MTX concentrations were expressed as picomoles/10^9 cells.

Results

Serum MTX concentrations at the time of investigation were below 1 nmol/l (i. e., the detection limit of the assay) in all the children. In 15 children MTX was found in the neutrophils in concentrations of 56–460 pmol/10^9 cells. In one patient no MTX was detected in the neutrophils separated on two different occasions. This child admitted to not having taken his medicine regularly.

Figure 1 shows the correlation between the weekly peroral dose of MTX and the MTX concentrations of the neutrophils. When all the data except for those relating to the child who had not taken the drug were included, a positive correlation between the weekly dose of MTX and the neutrophil MTX concentration was found ($r=0.51$, $P<0.01$), but a wide variation was observed.

Figure 1 also shows the results from repeated investigations in ten children, two of whom were studied after the MTX doses had been increased. In these two patients the new neutrophil MTX concentrations were higher after all three alteration of drug dose.

When seven children were studied at unchanged weekly MTX doses, three showed minor variations (1.5%, 5%, and 11%, respectively) in the neutrophil MTX concentrations, whereas the variations were considerably more marked (19%, 27%, 35%, and 40% respectively) in four children who had had relatively low neutrophil MTX concentrations at the first investigation.

The correlation between the MTX dose and the neutrophil MTX concentration was considerably closer ($r=0.70$, $P<0.001$) when only the higher value was used in the four children with relatively low neutrophil MTX concentrations at the first investigation.

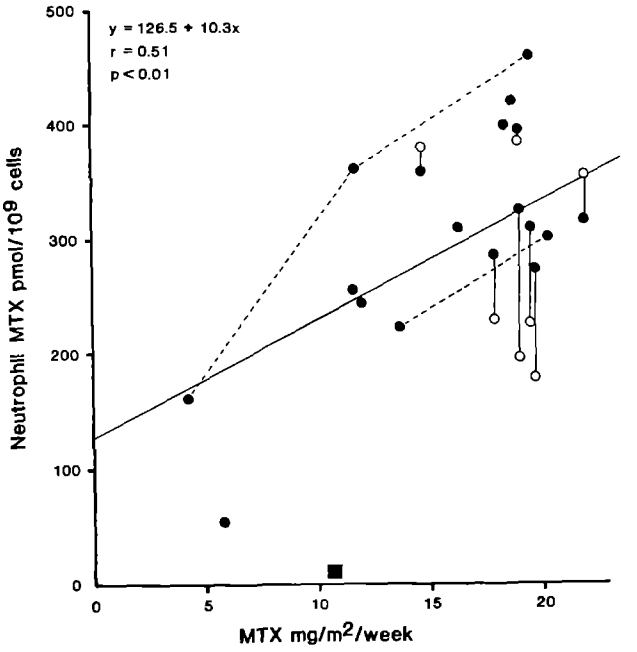

Fig. 1. Neutrophil methotrexate concentrations (neutrophil MTX) in relation to the weekly dose of MTX: ●----●, in two children examined after different weekly MTX doses; ○, result of first of two examinations of unchanged MTX doses, connected by *vertical bar* to result of second examination (●); ■, one child was examined twice. He admitted noncompliance

In 13 of the investigations the patients were found to be neutropenic (ANC < 1.5×10^9/l). No correlation was found between the dose of MTX and 6-MP and the ANC (data not shown).

In the patient sample as a whole MTX concentration did not correlate with the ANC, but in children with ANC > 1.5×10^9/l there was a significant negative correlation between the ANC and the neutrophil MTX concentration ($r=-0.71$, $P<0.01$; Fig. 2). The neutrophil MTX

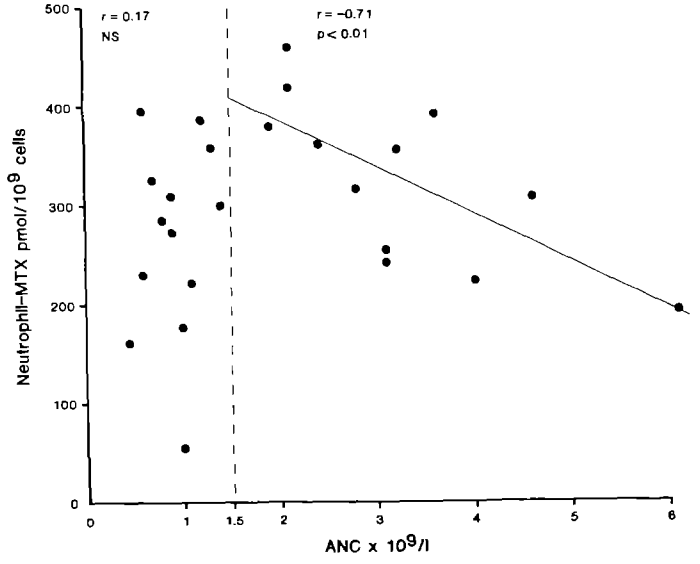

Fig. 2. Neutrophil methotrexate concentrations in relation to the absolute neutrophil count (ANC)

concentration was not higher in the neutropenic than in the non-neutropenic children, and it was not correlated with the ANC.

Discussion

Methotrexate probably exists in two forms in neutrophils, as it does erythrocytes [3]: a freely diffusable form, which follows the plasma MTX concentration curve [6, 10], and a slowly diffusable form, which has been incorporated into the myeloid bone marrow cells and reaches a peak concentration in the circulating neutrophils about 7 days after a 24-h MTX infusion [13]. This is the average time it takes for cells in the proliferating myeloid compartment to mature and be released into the circulation as neutrophils [2, 4]. The slowly diffusable MTX in myeloid cells is a result of intracellular metabolism of MTX to polyglutamate derivatives, which exist partly as free drug in the cytoplasm and partly bound to dihydrofolate reductase (DHFR) [1, 17].

MTX arrests the cell division in the S-phase of mitosis [16]. The intracellular drug concentration plays a role in the extent to which cell division is affected [5]. A test of the significance of the erythrocyte MTX concentration (ery-MTX) for the myelosuppressive action of MTX did not reveal any correlation between ery-MTX and ANC [14].

MTX concentrations in circulating neutrophils, however, might be expected to provide more insight into the intracellular pharmacokinetic basis of the neutropenia often encountered during maintenance therapy of childhood ALL with MTX and 6-MP. In the present study the MTX concentrations of the circulating neutrophils correlated with the weekly dose of MTX, but the correlation was weaker than in a former study of the relation between ery-MTX and MTX dose. One explanation for this may be that the erythrocytes are a slowly changing compartment for MTX, since erythrocytes containing MTX remain in the circulation for 2–3 months [15] whereas the circulating neutrophils have a half-life of about 8 h.

Another explanation for the relatively weak correlation between the MTX dose and the concentration of MTX in the neutrophils may be disturbances/variations of the proliferation and maturation times of the myeloid bone marrow cells, which are known to vary very widely even in non-treated persons [11]. This will cause the time at which the peak concentration of MTX occurs in the circulating neutrophils to vary. Examinations of neutrophil MTX in six psoriatic patients treated with low-dose MTX weekly and examined two or three times in a week between two doses of MTX showed that the time to peak MTX concentration in the circulating neutrophils varied widely (from the 2nd to the 7th day) after MTX administration, and that this variation was independent of the ANC (to be published). The present observation that the four children with the lowest neutrophil MTX concentrations had the greatest increase of the neutrophil MTX upon reexamination further supports the view that the time from MTX administration to neutrophil peak MTX concentration probably varies in these patients from week to week.

The maintenance treatment in our material resulted in neutropenia in about half the cases at the time of investigation. When the neutrophil MTX concentration for the patient sample as a whole was evaluated no correlation with ANC was found. The high MTX content of the neutrophils of the neutropenic children theoretically expected as an explanation for the neutropenia was not seen.

The observed inverse correlation between ANC and neutrophil MTX in the non-neutropenic children may be explained by a 'dilution effect'. Since the MTX content of the myeloid cells of the proliferating cell pool of the bone marrow is expected to be reduced by 50% by each cell division, a dilution effect will arise when MTX is incorporated in the bone marrow cells at times of augmented proliferative activity (e. g., as a response to infection) [2].

If the degree of neutropenia were directly correlated to the incorporation of MTX in the bone marrow cells expressed by the neutrophil MTX concentration 6–7 days after the last MTX dose, the regression line of Fig. 2 would be extrapolated to an ANC of zero. Consequently, the MTX concentration of the neutrophils of the neutropenic children should average 450 pmol/10^9 cells, which was not seen. The reason for this may be a longer myeloid proliferation and maturation time in these patients than in the non-neutropenic children [2]. This would give more time for the diffusable MTX fraction to leave the cells and result in a later and lower peak concentration of MTX in the circulting neutrophils. Another possible explanation might be that the isolated neutrophils represent the 'survivors' after the cytostatic drug therapy; they could have survived to be released into the circulation because their myeloid precursors for some unknown reasons incorporated too small an amount of MTX to kill the cell.

From our results we must conclude that the neutrophil MTX concentration measured 6–7 days after the last MTX dose did not explain the neutropenia seen in these children. The MTX concentrations measured in circulating neutrophils were a result of the variable granulopoietic behavior of these patients rather than explaining the myelosuppressive effect of MTX.

However, 6-MP is at least as myelotoxic as MTX. Neutropenia has been found to be correlated with the concentration of a metabolite of 6-MP (6-thioguanine) in erythrocytes [7]. The metabolism of this drug must also be taken into account in consideration of the pharmacologic basis of neutropenia occurring during maintenance therapy of childhood ALL.

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