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Flow cytometric analysis of marrow cell kinetics in children treated with high-dose MTX and CF rescue

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Summary. Normal marrow cell kinetics were studied by flow cytometry with computer analysis in 11 children with malignancies who received high-dose MTX followed by CF rescue. Nine children with hematological tumors in remission each received an infusion of MTX over 24 h, followed by delayed CF rescue. In 8 of the 9, an accumulation of cells in early to mid-S phase and a decrease of cells in G_2/M phase were observed at 24–48 h after the beginning of the MTX infusion. At 144 h after MTX infusion this kinetic perturbation disappeared and the DNA histogram returned to the same state as before therapy. Two children who had malignant bone tumors without marrow infiltration each received an infusion of MTX over 6 h with early CF rescue following an initial IV injection of vincristine. They did not have any prominent perturbation of marrow cell kinetics after MTX exposure, except for a transient increase of cells in G_2/M phase.

These results confirm that with the high-dose MTX therapy described above for hematological malignancies the impairment of marrow cell kinetics was much more severe and was soon followed by complete recovery, whereas with the therapy for solid tumors the impairment was much slighter.

Introduction

High-dose MTX therapy with CF rescue (HDM-CFR) has been widely used in the treatment of various childhood cancers [1], and it is considered as an antitumor therapy with comparatively moderate toxicity on bone marrow cells. However, excessive CF recue may attenuate the antitumor effect of MTX on hematological tumors, and reduced CF rescue may cause lethal bone marrow suppression. Therefore, it was deemed necessary to assess the in vivo effect of MTX on cytokinetics of human bone marrow cells. With the advent of DNA-specific fluorescent dyes [7] and the development of FCM, cell cycle distribution can be readily measured by FCM analysis of DNA content [8], and up to now, relatively few investigations have dealt with the cytokinetic effect of MTX on normal human marrow in clinical trials.

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Abbreviations: MTX, methotrexate; CF, citrovorum factor; FCM, flow cytometry

This study was designed to compare the cytokinetic effects of two regimens of HDM-CFR: delayed CF rescue for hematological tumors vs early and minimum CF rescue for solid tumors, on normal bone marrow cells in children.

Materials and methods

Patients and treatment regimen. HDM-CFR [9, 10] was performed in 11 patients and they and/or their parents consented to entry on this study. Table 1 shows clinical features and treatment regimens for the patients. The regimen with early CF rescue (referred to as regimen A from here onward) was performed in two children with an osteosarcoma. The regimen with delayed CF rescue (regimen B) was administered to nine children with ALL or malignant lymphoma who were in hematological remission: eight of the nine children have been disease-free since achievement of their first remission and only one, the youngest infant (case 11), has had hematological and CNS relapses. For regimen A, alkalinization with acetazolamide (Diamox) was started 12 h before infusion of MTX, concomitantly with a single dose of vincristine (2 mg/m^2). MTX 8000 mg/m^2 was infused over 6 h, followed by IV fluids for 48 h with sodium bicarbonate 33 mEg/l. CF was started 3 h after completion of the infusion, at a dose of 15 mg/m^2 IV every 3 h for a total of nine doses, followed by the same dose administered every 6 h for the next 48 h. For one child (case 1) one course of MTX infusion was given without an initial IV injection of vincristine. For regimen B, 2000–6000 mg/m^2 was infused over a period of 24 h. CF was started 12 h after termination of MTX infusion; doses of 15 mg/m^2 were given IV every 6 h for a total of seven doses. Alkalinization and hydration were performed as for regimen A. For all patients three courses of weekly MTX infusion were performed.

Flow cytometric measurement of DNA content of bone marrow cells.

In the patients treated according to regimen A, bone marrow aspirates, were obtained at −12, 6, 24, and 48 h (the first MTX infusion starting at time zero). In the patients treated according to regimen B the aspirates were obtained at 0, 24, 48 (72) and 144 h relative to the start of the first MTX infusion. In three patients, bone marrow samples were also obtained at 144 h after the start of the third (last) MTX infusion. Bone marrow cells were diluted with phos-

Table 1. Clinical features and plasma MTX concentrations of children treated with HDM-CFR

Patient no.	Initials	Sex	Age (years)	Tumor	Regimen	Dosage of MTX (/m²)	Plasma MTX concentrations (× 10^{-5} *M*)		
							6 h	24 h	48 h
1.	N. T.	M	10.5	Osteo-sarcoma	A	8000	64.0	0.32	0.038
2.	M. Y.	F	15.6	Osteo-sarcoma	A	8000	76.0	0.28	0.020
3.	I. R.	F	11.1	ALL	B	3000	7.8	7.3	0.045
4.	F. K.	F	6.2	ALL	B	2000	4.6	4.6	0.046
5.	I. M.	M	4.9	ALL	B	2000	25.0	5.2	0.028
6.	S. T.	F	6.0	ALL	B	2000	4.9	5.0	0.040
7.	U. S.	F	7.3	ALL	B	2000	2.4	5.9	0.040
8.	K. D.	M	10.4	NHL	B	6000	22.0	64.0	0.025
9.	S. A.	M	4.9	NHL	B	4500	7.0	16.0	0.041
10.	H. R.	F	8.7	ALL	B	2000	5.7	8.9	0.049
11.	O. M.	F	1.4	ALL	B	6000	7.6	7.5	0.036

ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin's lymphoma

phate-buffered saline (PBS) and centrifuged through a Ficoll-Conray mixture for 30 min at 400 *g*. The mononuclear cells harvested were washed twice in PBS and the cell pellet was resuspended in 0.5 ml hypotonic propidium iodide solution (P.I. 5 mg + trisodium citrate 100 mg + Nonidet P-40 0.1 ml/DW 100 ml). Then 0.5 ml RNase (20, 713 units/ml, Worthington Biochemical, Freehold NJ) was added and incubated for 30 min at room temperature. The stained suspensions were filtered through a 44 μm nylon mesh just prior to FCM. An FACS 440 flow cytometer equipment with a 50-μm-diameter nozzle (Becton-Dickinson FACS Systems, Calif) was used for DNA content and light scatter measurements. A 488-nm argon ion laser line at 300 mW was used for excitation, and the total fluorescent emmission above 590 nm was measured. At least 30 000 cells were counted in each sample. The DNA histogram was analyzed by Fried's program [3] on a Hitac M-260 D system computer (Hitachi, Japan).

Plasma MTX concentration. Blood samples were drawn at 6, 12, 24, 48, and 72 h after the beginning of the MTX infusion for evaluation of MTX pharmacokinetics. Concentrations of MTX were measured by an enzyme inhibitory assay [6].

Mitotic index. Bone marrow cells were stained with a May-Grünwald-Giemsa solution. The percentage of mitotic figures per 2000 nucleated cells was recorded as the mitotic index (MI).

Results

Cell cycle

Regimen A: Figure 1 shows DNA histograms of nucleated bone marrow cells for one patient (case 1) treated according to regimen A. The DNA histograms did not show considerable changes except some increase in the proportion of cells in G_2/M phase at 6 h after the beginning of the MTX infusion. The histograms of the other patient (case 2) were similar to those of patient 1. Patient 1 received one MTX infusion without an initial vincristine injection. With this infusion the DNA histogram at 24 h after the beginning demonstrated a considerable accumulation of cells in early and mid-S phase.

Regimen B: In eight of nine children treated according to regimen B, bone marrow cells exposed to MTX showed an appreciable cytokinetic perturbation, and the pattern of the perturbation was uniform in all eight children. Figure 2 shows a representative sequential change in DNA histogram after MTX exposure. At the end of a 24-h MTX infusion an accumulation of cells in early S phase was observed, with a corresponding decrease in the number of cells in the late S and G_2/M phases. At 24 h after the termination of MTX infusion (12 h after initiation of CF rescue) the number of cells in G_2/M phase was still lowered, and the proportion of cells in S phase continued to increase, with an accumulation of cells in mid-S phase. In three children bone marrow cells were also obtained at 72 h after the start of MTX infusions. They showed only a slight accumulation of cells in early S phase and decrease of cells in G_2/M phase (data not shown). At 144 h after the initiation of MTX infusion (24 h before the start of the next infusion), the histogram was similar to that just before first MTX infusion. The change in the distribution of cells through the cell cycle is illustrated in Fig. 3. The percentage of cells in S phase increased, reached a peak at 48 h, and returned to control values by 144 h. There was a small decline in the proportion in G_1 at 24–48 h, and a significant reduction in the proportion of cells in G_2/M at 24 h.

To ascertain whether weekly infusions of MTX have cumulative effect on marrow cell kinetics or not, we analyzed three patients' DNA histograms for marrow cells sampled 144 h after the start of the third (last) MTX infusion. None of the histograms showed any significant change compared with those before therapy. In contrast to the above eight children, patient 11's histograms were not significantly different after exposure to MTX.

MI and growth fraction

Figure 4 shows the changes in the percentages of cells in S phase and mitotic cells in total nucleated marrow cells in eight children who showed considerable cytokinetic changes when treated according to regimen B. At 24 h af-

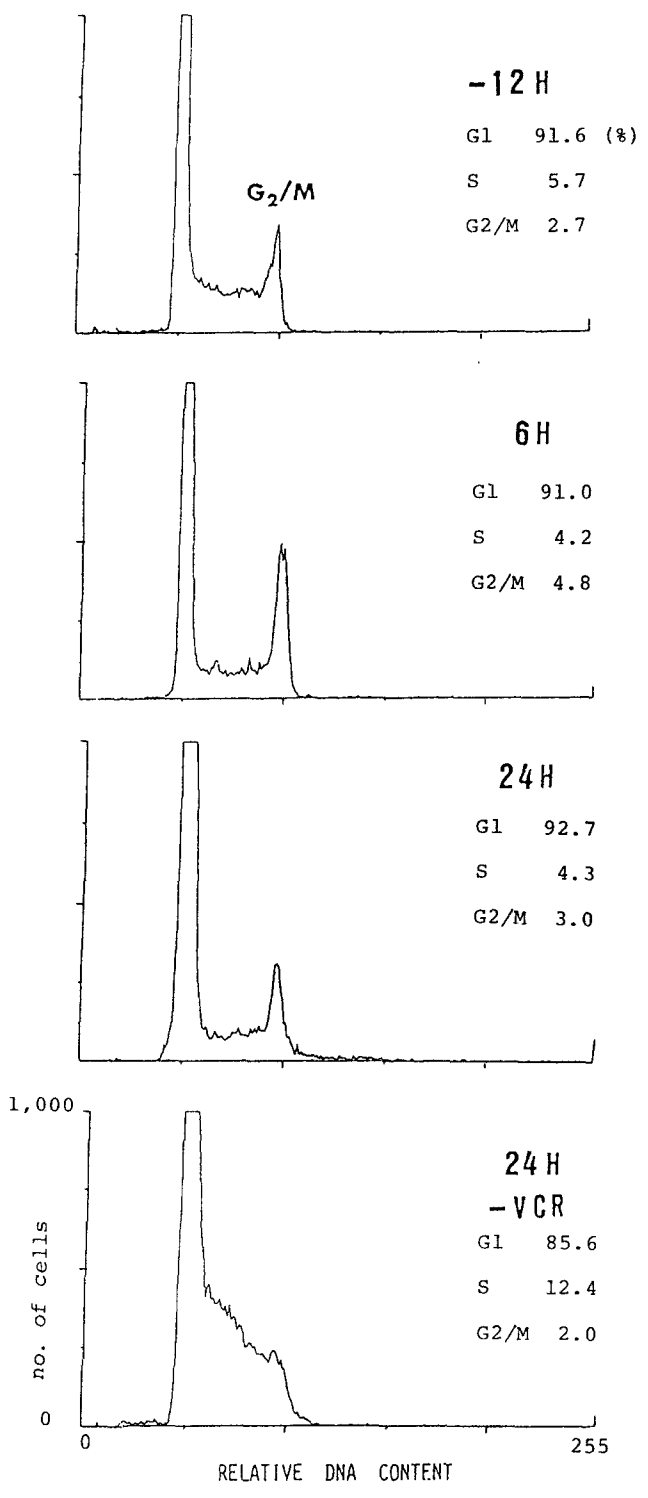

Fig. 1. Sequential changes in DNA histograms obtained for normal bone marrow from a patient (case 1) treated according to regimen A. The *top histogram* represents was drawn with reference to cells just before the initial vincristine injection, and the *bottom histogram*, after MTX infusion without initial vincristine injection

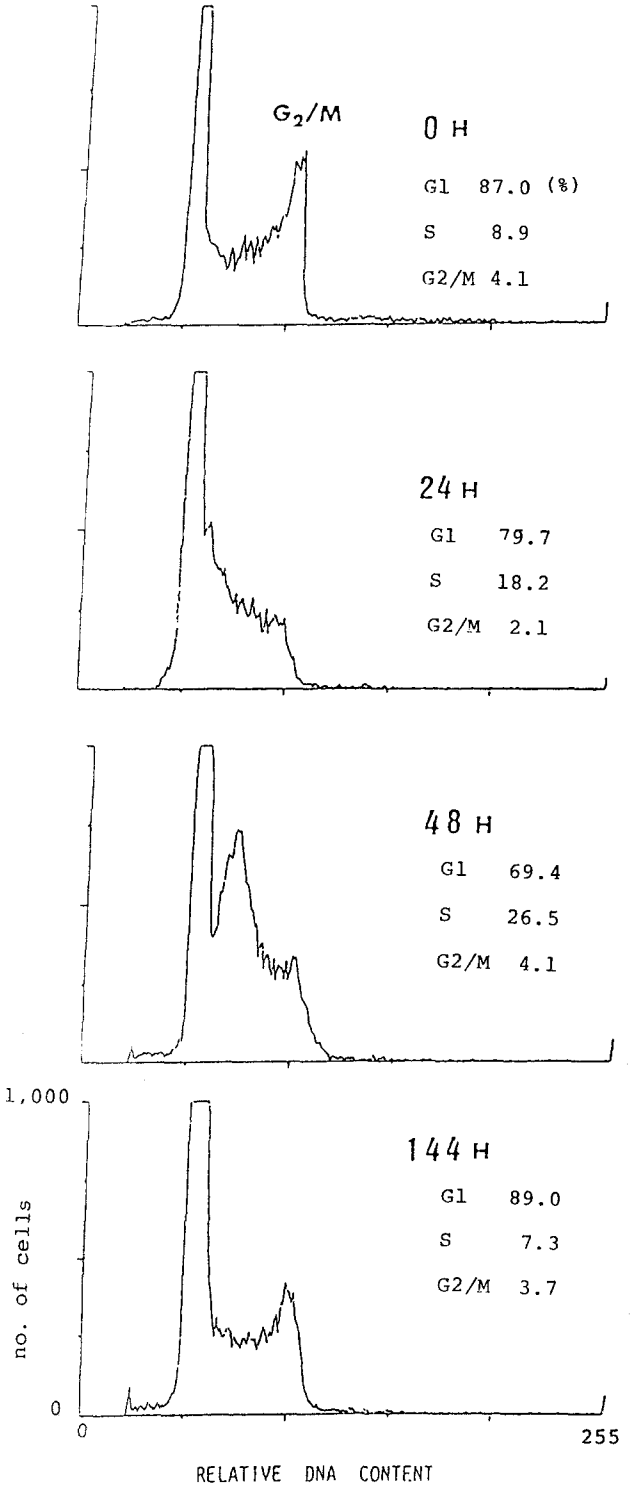

Fig. 2. Changes in DNA histograms of representative marrow samples from patients treated according to regimen B

ter the start of an MTX infusion the percentage of S phase cells was increased and remained elevated for 24 h. At 144 h after the start of the infusion it had returned to the same value as before treatment. The MI promptly fell and then began to increase, but had not completely recovered by day 6.

Plasma MTX level

The doses given to the patients are compiled in Table 1, which shows that the 48-h MTX plasma level is constant regardless of the dose and duration of infusion of MTX. With regimen B the plasma MTX levels at 6 and 24 h after the start of infusion in case 11 were 7.6×10^{-5} *M* and 7.5×10^{-5} *M*, respectively, and they were higher than the

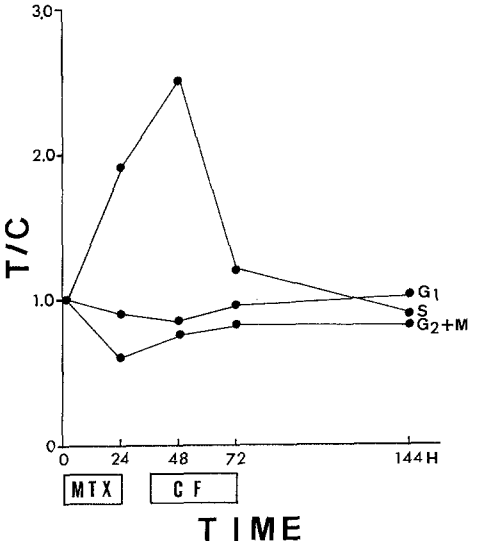

Fig. 3. The effect of MTX on cell cycle transit. Data from eight patients (case 3–10) treated according to regimen B were averaged for each time point and are plotted as ratios of post-treatment (T) to control (c), values

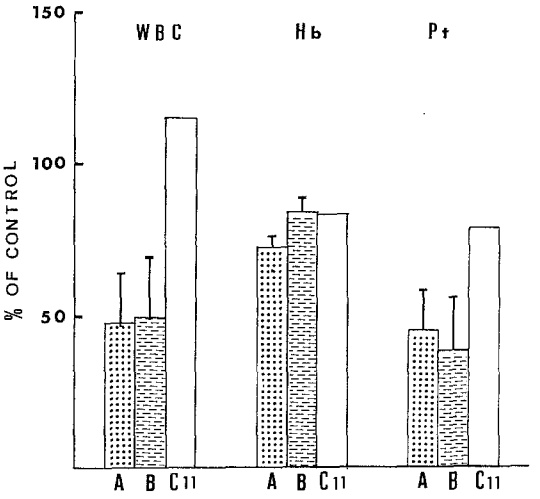

Fig. 5. The effect of MTX infusion on the number of blood cells. Each blood cell was evaluated by comparing the number of blood cells at the nadir after infusion with that before infusion (mean ±SD). *A*, regimen A; *B*, regimen B (except case 11); *C 11*, case 11; *WBC*, white blood cells; *Hb*, hemoglobin concentration; *Pt* platelet count

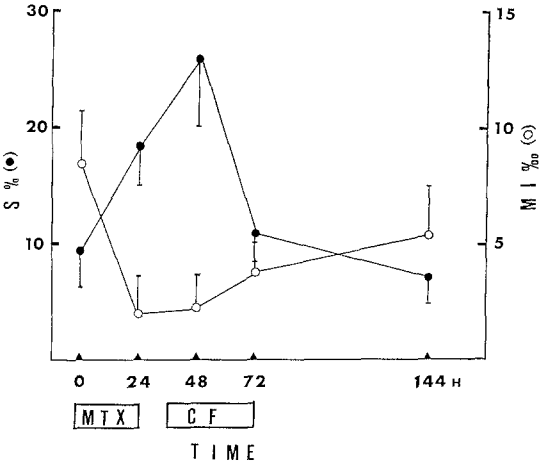

Fig. 4. Effect of MTX infusion on MI and percentage of cells in S phase. Data from eight patients (cases 3–10) in regimen B were averaged for each time point (±SD)

median values (6.3×10^{-5} *M* at 6 h and 6.6×10^{-5} *M* at 24 h) for the other eight children, for all of whom considerable changes were seen in the histograms.

Effect on number of peripheral blood cells

Considerable decreases in the WBC and platelet counts were observed both in patients who received regimen A and in those who received regimen B. The range of percentage decrease in the number of WBC was similar with both regimens (Fig. 5). Significant fall of WBC, was observed soon after MTX infusion with regimen A, whereas with regimen B it was not observed until about 10 days after the infusion. The youngest infant treated (case 11) did not have any decrease in WBC. The point at which the platelet count began to fall was variable in the entire population of children and was not dependent on regimen. Mild anemia was observed in all children with both regimens (Fig. 5).

Discussion

It is well known that younger patients tolerate HDM-CFR regimens better than older patients [12, 13]. Of the toxicities after high-dose MTX infusion, myelosuppression is the most important, limiting repeated infusions of MTX. Nonetheless, we have not been informed of the in vivo cytokinetic effects of HDM-CFR regimens on normal bone marrow cells in children, though a few studies with adult patients have been reported [5, 11]. Our present results demonstrate that the HDM-CFR regimen used for hematological malignancies (regimen B) caused a marked change in DNA histograms at 24–48 h after the start on an MTX infusion, which returend to the pretreatment state by day 6 after infusion in all patients except the youngest one. Flow cytometric analysis revealed an accumulation of cells in early or mid-S phase, and this finding was consistent with MTX-induced inhibition of passage through the S phase. The decrease in the number of cells in G_2/M phase with reduced MI suggested cell death or exit from these compartments. The pattern of cell cycle perturbation was similar to that reported by other workers for adult patients [5, 11].

DNA histograms of bone marrow cells obtained in patients who received regimen A showed no remarkable change except an accumulation of cells in G_2/M phase at 6 h after the start of MTX infusion. This accumulation consists in metaphase arrest by vincristine. Is absence of inhibition of S phase transit on cycle progression due to a difference in regimen (short exposure time to MTX or early CF rescue) or initial IV injection of vincristine? Analysis of the histograms for regimen A without initial vincristine injection clearly showed that the absence of inhibition of S phase transit was caused by the initial vincristine injection and not by either short exposure time to MTX or early CF rescue. Interaction of MTX and vincristine has been observed and extensively studied in various tumor cells in vitro, and it has been postulated that vincristine may increase the antitumor activity of MTX [4]. However,

this effect has been described as unlikely in normal cells [2]. Our results suggest that pretreatment of vincristine masked MTX-induced kinetic perturbations of normal bone marrow cells. Thus the effect of each drug on the antitumor activity of the other in vivo remains to be determined.

In general, with a similar MTX dosage, younger children have lower plasma MTX concentrations than older children or adults, and the toxicity after high-dose infusions is not dose-related but age-related [12]. In fact, almost all studies with MTX in pediatric age groups have been conducted in children beyond the age of infancy, and the pharmacokinetics of MTX in neonates and young infants remains unclear. The youngest infant (case 11) in our study did not have a significant cytokinetic change in bone marrow cells after MTX infusions, although she had higher plasma MTX concentrations, than the median values for the other eight children treated according to regimen B. We not know whether this less severe toxicity of MTX to bone marrow cells is common to children still classifiable as infants or whether it had to do with the particular patient. The above questions cannot be resolved without further investigations in much younger children.

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