Differential Effect of High-Dose Methotrexate on Erythropoiesis and Granulocytopoiesis in Humans

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ABSTRACT

The kinetics of erythroid and granulocytopoietic cell production were investigated during Courses 1 and 4 of high-dose methotrexate therapy with citrovorum factor rescue in a patient suffering from metastatic osteogenic sarcoma. Using the technique of quantitative ^{14}C autoradiography, relative production rates were determined before, as well as 2, 24, 48, and 72 hr after, methotrexate infusion. There was only a minor decrease of the relative granulopoietic cell production 2 hr after methotrexate infusion followed by an overshoot reaction after 48 hr with a maximum of 3 to 4 times the pretherapeutic value. The relative erythropoietic cell production dropped to less than one-third of the pretherapeutic level during both courses and remained low during the period of postinfusion observation. The results indicate a severe and long-lasting impairment of the erythropoietic cell series, which is likely to include the committed stem cell pool. The impairment of granulocytopoiesis was much smaller and was followed quite soon by a reaction of recovery. The rate of DNA synthesis of individual cells was subnormal in all cell types investigated prior to Course 4 and was hardly affected by the methotrexate. Intracellular accumulation of methotrexate polyglutamates and differences in this pattern of accumulation between the red and white cell series are discussed as one possible explanation in this context.

INTRODUCTION

HDM-CFR[2] is considered an antitumor therapy with comparatively moderate toxicity on the bone marrow cell systems. It has been shown (19) that human bone marrow in vitro colony-forming units in agar culture grown in MTX concentrations of up to 1 mM do not significantly decrease in number if the exposure is kept within a limit of 24 hr. On the other hand, a significant decrease of the ratio of erythropoietic to granulocytopoietic cells in the bone marrow 2 days after the start of the HDM-CFR has been reported (23). Since the method of quantitative ^{14}C autoradiography offers the possibility to study kinetic changes even in small compartments of human bone marrow cells, this study was designed to detect differential effects on the RBC and WBC lineage. The bone marrow was investigated during 2 courses of HDM-CFR in a patient suffering from metastatic osteogenic sarcoma.

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[2] The abbreviations used are: HDM-CFR, high-dose methotrexate therapy with citrovorum factor rescue; MTX, methotrexate; dUrd, deoxyuridine; dThd, thymidine; dCyd, deoxycytidine.

MATERIALS AND METHODS

The patient was a 47-year-old man whose left leg had been amputated because of an osteogenic sarcoma 21 months before and who without any preceding antitumor therapy exhibited extended lung metastases upon admission. Ten consecutive courses of HDM-CFR were instituted, and the present cell kinetic analysis was performed during the first (termed Course A in the following) and fourth course (Course B) of drug administration. In Course A 2.0 and in Course B 7.5 g of MTX per sq m were infused over a period of 6 hr following an initial i.v. injection of 2 mg vincristine. Leucovorin rescue started 2 hr after termination of the MTX infusion and was continued for 72 hr. Doses of 15 mg were given i.v. every 3 hr over the first 24 hr and were followed by the same doses administered every 6 hr. Between Courses A and B, there was an interval of 63 days. During this period, the general status of the patient was stable, and no significant change in the pulmonary tumor mass was observed. The pretreatment data of peripheral blood cells were within the normal range but showed a decreasing tendency (Course B values in parentheses): hemoglobin, 15.4 (13.8) g/dl; leukocytes, 10,000 (5800)/μl; platelets, 232,000 (237,000)/μl. The data on MTX serum concentrations and the quotients of dUrd and dThd incorporation in the bone marrow have been reported in a previous paper (21) and are briefly summarized in Table 1.

The method of quantitative ^{14}C autoradiography for determining the DNA synthesis rate of individual cells has been described in detail in previous papers (7, 9). Its application for estimating cell production rates in individual morphological bone marrow cell compartments is further explained elsewhere (12). Bone marrow cells obtained by sternal or iliac crest puncture were suspended and washed twice in TC Medium 199. A single-cell suspension was prepared by passing the samples through a glass pore filter with a pore size between 45 and 90 μm. The cells were incubated in the presence of 5 μM 5-fluoro-2'-deoxyuridine and 10 μM dCyd for 6 min in a shaking water bath at 37.2°. Subsequently, [^{14}C]dThd was added to provide a final concentration of 10 μM, and the incubation was continued for either 3 or 7 min and stopped by placing the incubation tubes into ice water. 5-Fluoro-2'-deoxyuridine is used in order to block the endogenous synthesis of TMP and thereby to prevent a dilution of the specific activity of dThd in the TMP pool. By the addition of dCyd, a possible depletion of the dCTP pool due to the presence of 10 μM dThd can be prevented (2). One extra tube was incubated with [^{3}H]dThd over a period of 15 min in order to provide autoradiographs for labeling index determinations. Smears were prepared on microscopic slides, fixed in absolute methanol for 48 hr, and provided with radioactive reference sources consisting of [^{14}C]methylmethacrylate. Autoradiographs were prepared using AR.10 stripping film and evaluated quantitatively by means of a computer-assisted microscopic grain counter (13). Since the incubation procedure permits only labeled dThd to enter the TMP pool, the dThd incorporation rate becomes equivalent to the DNA synthesis rate. The quantity of radioactivity incorporated into the DNA of single cells is determined from the grain counts by means of the reference blackening produced by the radioactive standards. This procedure provided the DNA synthesis rate per individual labeled cell in a morphologically defined compartment.

The relative number of cells in a morphological bone marrow compartment was determined from Pappenheim-stained smears immedi-

ately prepared after bone marrow puncture. DNA synthesis times were derived for the various morphological compartments by dividing the amount of thymine per diploid human cell by the autoradiographically determined rates of dThd incorporation into DNA; 6.3×10^{-15} mol of thymine have been determined biochemically in the DNA of diploid human cells (7, 8). This amount is doubled during the period of DNA synthesis which allows, according to the rate of DNA synthesis, a computation of the DNA synthesis time in a compartment.

The relative cell production rates of the RBC and WBC series were computed by multiplying the relative cell number in a morphological compartment by the labeling index and dividing the product by the DNA synthesis time of that compartment. The production rate in a cell lineage is the sum of the production rates of the individual compartments of that lineage. The figures are expressed in terms of cells produced per hr per 1000 nucleated bone marrow cells. In some instances after MTX therapy, there were too few pro- and basophilic erythroblasts in the smears in order to perform an analysis. Since in normal bone marrow the ratio of production rates of proerythroblasts: basophilic erythroblasts:polychromatic erythroblasts is 1:2:4.8 (7), the production rate of polychromatic erythroblasts was doubled in these instances to provide an estimate for the whole erythroid cell system. This is more likely to represent an overestimate rather than an underestimate of the actual rate of total erythroblast production.

RESULTS

Table 1 summarized data on the MTX concentrations and

the quotients of the dUrd and dThd incorporation rate during Courses A and B. In Table 2, the rates of DNA synthesis per single bone marrow cell in the various morphological compartments are listed for both courses. In Course A, prior to the onset of therapy, slightly increased values were seen in the myeloid and decreased values in the erythroid series. The latter is in keeping with previous findings in anemias of chronic diseases (11). Prior to Course B, all figures were significantly below the normal level; however, this effect was much more pronounced in the RBC than in the WBC series. After MTX application, the myeloid series showed an initial depression in both courses which after 48 hr was followed by an overshoot reaction in Course A and by a tendency towards normalization in Course B. The DNA synthesis rate of the RBC series varied only slightly during Course A and showed a further decrease from low pretherapy values in Course B.

The relative cell production rates are plotted in Chart 1 for both courses of treatment. Prior to drug administration, the relative cell production rate was roughly normal in both instances. In healthy individuals, relative rates of 5.9 cells/hr/ 1000 nucleated bone marrow cells were determined in the erythroid series and 6.1 in the granulocytic series (12). In both
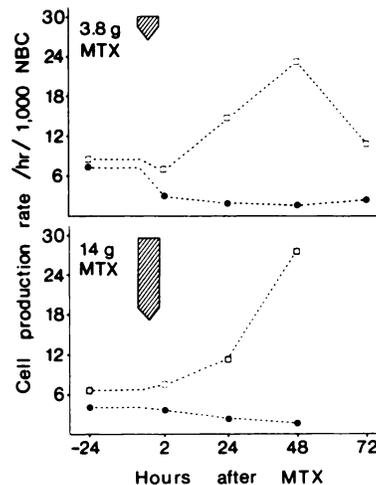
Table 1

MTX concentrations and quotients of dUrd and dThd incorporation

MTX concentrations in the serum during Courses A and B of HDM-CFR are expressed in terms of μM. These values as well as the quotients of the incorporation rates of 10 μM dUrd and 10 μM dThd have been compiled from a previous study (21).

	MTX serum concentration (μM)	dUrd:dThd
Course A		
Pretreatment value	0.0	1.21
0.1 hr post-MTX	64	ND[a]
24 hr post-MTX	0.85	0.14
48 hr post-MTX	0.1	0.81
72 hr post-MTX	0.07	0.95
Course B		
Pretreatment value	ND	0.97
0.1 hr post-MTX	213	ND
2 hr post-MTX	ND	0.04
24 hr post-MTX	0.8	0.25
48 hr post-MTX	0.2	0.90

[a] ND, not done.

Chart 1. Relative rates of erythropoietic (●) and granulocytopoietic (□) cell production during the first (*top*) and fourth (*bottom*) course of HDM-CFR, plotted against the time after termination of the 6-hr infusion of MTX.

Table 2

DNA synthesis rates of various types of bone marrow cells during HDM-CFR

Rates of DNA synthesis are expressed in terms of $10^{-18} \times$ mol per min per labeled cell, in the various morphological bone marrow cell compartments during Courses A and B of HDM-CFR. The normal values have been taken from previous studies (3, 7).

	Rate of DNA synthesis ($10^{-18} \times$ mol/min/labeled cell)					
	Proerythroblasts	Basophilic erythroblasts	Polychromatic erythroblasts	Myeloblasts	Promyelocytes	Myelocytes
Course A						
Pretherapy	9.33 ± 0.54[a]	7.62 ± 0.24	3.57 ± 0.18	10.05 ± 0.59	10.08 ± 0.32	8.63 ± 0.57
2 hr post-MTX			3.71 ± 0.44		6.01 ± 0.39	6.39 ± 0.30
24 hr post-MTX			2.52 ± 0.24	7.74 ± 1.41	9.35 ± 0.67	7.55 ± 0.43
48 hr post-MTX			4.17 ± 0.50	13.09 ± 2.33	11.81 ± 0.56	10.18 ± 0.38
72 hr post-MTX			3.76 ± 0.27	7.17 ± 0.45	5.54 ± 0.19	4.76 ± 0.15
Course B						
Pretherapy	5.54 ± 0.39	3.68 ± 0.17	2.64 ± 0.11	5.65 ± 0.41	5.31 ± 0.22	5.19 ± 0.16
2 hr post-MTX	5.67 ± 0.30	3.48 ± 0.16	2.12 ± 0.09	5.0 ± 0.21	4.54 ± 0.13	4.17 ± 0.12
24 hr post-MTX			2.40 ± 0.31	6.61 ± 0.63	6.18 ± 0.24	5.45 ± 0.23
48 hr post-MTX			1.83 ± 0.17	7.60 ± 0.67	7.17 ± 0.28	5.73 ± 0.18
Normal (n = 5)	11.2 ± 2.3	9.5 ± 2.0	6.2 ± 1.2	8.0 ± 1.1	7.8 ± 0.5	7.5 ± 0.6

[a] Mean ± S.E.

instances, granulocytopoiesis showed little or no decrease in the rate of relative cell production following HDM-CFR, whereas there was an immediate and long-lasting drop in the erythroid series. Forty-eight hr after termination of the MTX infusion, the relative granulocytic cell production rate was increased by a factor of 3 to 4 over the pretreatment value. It is obvious from Table 2 that in Course B there was only a moderate change of the DNA synthesis rate contributing to this increase. The major factor was a rise of the relative number of myeloid cells in S phase. Whereas the pretreatment figures for S-phase cells per 1000 nucleated bone marrow cells in Course B were 130 in the erythroid and 138 in the myeloid series, the corresponding figures 48 hr post-MTX amounted to 52 and 424, respectively. The myeloid series apparently responded by a partially synchronized wave of proliferation. In Course A, on the other hand, changes of the DNA synthesis rate were significantly more involved in producing the same type of proliferative wave.

DISCUSSION

An obvious divergence of the relative production rates of RBC and WBC precursors in human bone marrow has been observed during the initial 72 hr of HDM-CFR. With the applied technique of quantitative ^{14}C autoradiography, no information is obtained on the absolute values of bone marrow cell production which may have been grossly different during Courses A and B. The absolute number of bone marrow cells obtained by aspiration varies due to technical factors and depends on the site of aspiration. Therefore, cell production rates in the present study were always referred to a fixed number of 1000 nucleated bone marrow cells. Some information, however, on total cellularity after MTX administration is provided by animal studies. After injection of 60 mg MTX per kg into C57BL mice, Vogler et al. (25) observed a reduction of total cellularity in the femora amounting to roughly 50% on Days 2 and 3. Therefore, the overshoot of the relative granulocytic cell production currently observed after 48 hr of HDM-CFR (Chart 1) is likely to balance more or less the reduced total cellularity. There is thus some immediate equilibrium reaction in the rate of absolute granulocytic cell production. Concurrently, however, the absolute rate of erythroid cell reproduction is possibly even more depressed than is made evident by the relative rates.

This view of a differential effect of MTX is in agreement with differences in the repopulating ability of granulocytic and erythroid cells after MTX observed in rats (5, 6). It is further substantiated in patients under HDM-CFR by a ferrokinetic study showing a dose-dependent reduction of the plasma iron turnover and of the RBC iron utilization rate down to MTX serum levels of 0.05 μM (22). A decrease of the ratio of erythropoietic to granulocytopoietic cells in the bone marrow from 0.78 on Day 0 to 0.14 on Day 2 during 5 initial courses of HDM-CFR has been observed by Schreml and Lohrmann (23). In accordance with data from human in vitro colony-forming units in agar culture under MTX (19), the early response of the relative granulocytic cell production rate indicates little or no impairment of the unrecognizable granulocytic precursor cells. On the other hand, the immediate and lasting depression of the relative erythroid cell production rate points to an impairment of both the recognizable and unrecognizable precursor pool. According to all these data, a higher toxicity of MTX on the erythroid cell lineage is most likely.

Whereas the rates of relative cell production fall within the framework of anticipated results, the underlying DNA synthesis rate data of individual cells are somewhat unexpected (Table 2). First of all, roughly normal rates of DNA synthesis were only measured prior to Course A. The depressed rates of DNA synthesis prior to Course B were compensated in the granulocytic series by a higher percentage of proliferative cells in the bone marrow differentials leading to a roughly normal relative production rate. This was not quite the case in the erythroid cell lineage where a figure of 30% below the normal rate of erythrocyte production was observed. The a priori low DNA synthesis rates of individual cells in Course B were not markedly depressed any further by the action of MTX. On the other hand, polychromatic erythroblasts in both courses continued to synthesize DNA at significantly reduced rates when the MTX serum levels had fallen below concentrations associated with a significant decrease of the ratio of dUrd to dThd (Table 1).

Two lines of interpretation are conceivable in the discussion of these results. The first one is related to individual conditions in the patient presently studied. Prior to Course A, the erythroid cells, especially polychromatic erythroblasts, synthesized DNA at a lower rate than normal. The reduction is more pronounced than it was in 2 previously studied patients with malignant disorders (11). There is no information as to whether changes in the erythroblast DNA synthesis rate might be related to the stage of disease, i.e., to the metastatic spread or the tumor load. If there is such a direct influence, the effect of MTX was probably superimposed on an impairment caused by the malignant disease. However, this does not sufficiently explain the differences between Courses A and B detected for the DNA synthesis rates in both cell lineages (Table 2).

The other line of interpretation is related to the metabolism of MTX. It has been shown that MTX bound to polyglutamate is retained within liver cells and erythrocytes for weeks (1, 15, 26). The polyglutamate forms of MTX accumulated within these cells have been reported to be as active inhibitors of dihydrofolate reductase as MTX (18). Furthermore, the intraerythrocytic level of MTX conjugates increases with every new course of drug application in HDM-CFR.[3] In view of this accumulation, it is conceivable that the rates of DNA synthesis prior to Course B were still immediately affected by MTX. Differences in the accumulation pattern have been described for different tissues (26) and may also exist between the RBC and WBC series. They are possibly responsible for the differential effects on the relative cell production rates observed in the present study as well as on the repopulating ability of normal rat bone marrow as demonstrated by others (5, 6). These latter studies on normal tissue indicate that the individual conditions of the patient do not account primarily for the diverse response to MTX of erythro- and granulocytopoiesis.

The actual in vivo rates of DNA synthesis are reflected by the in vitro method that is currently applied only if after MTX application enough DNA catabolites are available in vivo to compensate for the block of endogenous TMP supply. As such, the presence of MTX in concentrations above 1 μM is not critical for the maintenance of a normal DNA synthesis rate as long as enough salvage pathway metabolites are available. This has been investigated in different cell systems including the bone marrow (4, 20, 24). Evidence for continuing DNA

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[3] A. Schalhorn, unpublished observation.

synthesis even after 24 hr of high-dose MTX infusion without any rescue has been provided by [^{3}H]dCyd incorporation studies into total acid-insoluble material which still yielded 24.6% of the pretherapy value in one study (17) and 28% in another (16). In normal basophilic and polychromatic erythroblasts of the rat, some 40 to 60% of DNA thymine is recruited from dead cells (14). On the other hand, 53% of DNA thymine in basophilic erythroblasts and 40% in polychromatic erythroblasts of the rat was found to stem from the exogenous supply when 10 μM of [^{14}C]dThd were administered (10). This dThd concentration, in turn, is sufficient to maintain an undisturbed rate of DNA synthesis when the endogenous pathway of TMP supply is completely blocked (9). These arguments favor the assumption that the reduced rates of DNA synthesis, as measured under the currently applied HDM-CFR regimen, could be maintained *in vivo* by virtue of the DNA breakdown products.

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