Effect of High-Dose Methotrexate with Citrovorum Factor on Human Granulopoiesis

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ABSTRACT

The present studies were performed to delineate the effect of high-dose methotrexate with citrovorum factor rescue on the measurable compartments of human granulopoiesis, including peripheral blood cells and bone marrow stem cells committed to granulopoiesis-monocytopenia. Parameters of granulopoiesis were determined during the first course of high-dose methotrexate with citrovorum factor rescue in five patients, and during four consecutive courses in one patient. The remarkably mild toxicity of high-dose methotrexate with citrovorum factor rescue reported in clinical studies was substantiated on the level of the committed stem cell compartment. The data suggest a higher sensitivity of in vitro colony-forming units in agar culture as compared to the more mature granulopoietic cells and document the exquisite sensitivity and regenerating capacity of erythroid precursors after this type of chemotherapy.

INTRODUCTION

The administration of a cytostatic drug, followed by its antidote, may selectively rescue normal cells, thereby abolishing undesirable side effects, while the antitumor effect is maintained. Based on experimental work by Goldin et al. (9), the technique of HDM-CFR has been introduced into clinical studies. HDM-CFR has been reported to be effective in osteosarcoma (13), refractory acute leukemia (6), and other solid tumors (7, 14). The pharmacology of HDM-CFR has been intensively explored (10, 32), but the mechanism of the differential sensitivity of neoplastic and normal cells under the condition of pharmacological rescue has not been fully elucidated.

The lack of serious side effects is a noteworthy fact in cases of effective rescue. The hematopoietic toxicity of HDM-CFR has usually been mild (27, 32), although severe myelosuppression has been encountered occasionally. However, detailed information on the changes of human bone marrow after HDM-CFR has not been reported. Since it is known that peripheral blood cells and recognizable bone marrow compartments may be nearly normal despite definite damage to the stem cell compartments after irradiation (19) and chemotherapy (24, 34), such information would be desirable to help to evaluate the toxic potential of HDM-CFR. The present studies were performed to delineate the reaction pattern in the different compartments of granulopoiesis after HDM-CFR, including hematopoietic stem cells committed to granulopoiesis (CFU-c).

MATERIALS AND METHODS

In 5 patients, the changes of hemopoiesis after the first course of HDM-CFR were studied. In 3 patients, HDM-CFR was part of an adjuvant treatment after surgery for osteogenic sarcoma, which was performed according to the treatment scheme given by Rosen et al. (29). One patient was treated for metastatic osteogenic sarcoma, and the fifth patient was treated for metastatic synovioma. The patients had not been treated with chemotherapy or radiotherapy prior to these studies except for the patient with synovioma, who had local irradiation and various types of chemotherapy until 16 months prior to HDM-CFR.

In one other patient under adjuvant chemotherapy for osteogenic sarcoma, the second, third, and fourth course of HDM-CFR were also studied for hemopoietic changes. Between these courses, cyclophosphamide and Adriamycin were given (29).

HDM-CFR was administered as described by Jaffe et al. (13). Vincristine (1.4 mg/sq m) was given 2 hr before the 6-hr MTX infusion to increase cellular uptake of MTX (11). Starting 2 hr after the MTX infusion, citrovorum factor (15 mg/sq m) was given every 6 hr i.v. The 48-hr and 60-hr MTX levels were utilized to guide duration of administration of citrovorum factor (31). Hydration, urinary flow, and urinary pH were monitored throughout the therapy period.

The methods used in our laboratory for studying chemotherapy-induced changes of granulopoiesis have been published previously (15, 16, 18). To summarize briefly, the following data were obtained serially before, during, and after HDM-CFR: WBC and differential counts; functional bone marrow PMN reserve measured as the maximal increment of peripheral blood PMN after rapid i.v. infusion of 200 mg hydrocortisone (4); bone marrow differentials obtained by counting at least 300 cells in Pappenheim-stained smears; stem cells committed to granulopoiesis-monocytopenia (CFU-c) in peripheral blood and bone marrow, assayed in a single layer agar culture system with human placenta-conditioned medium as a source of stimulating activity (16); proliferative activity of bone marrow CFU-c determined by [3H]dThd suicide (15).

Serum levels of methotrexate were determined by Wilab Scientific Laboratories, Baiersdorf, Germany, using the radioimmunoassay described by Raso and Schreiber (28). The lower limit of detection with this method is 5 × 10^-9 M.

For statistical analysis, the 2-tailed Mann-Whitney U test (20) was applied. Since this nonparametric rank test may err when the number of values and the differences are low, the Student t test was also performed on the [3H]dThd suicide data for confirmation of the U test. The application of this parametric test was based on the following considerations. The [3H]dThd data obtained from a larger group of patients with adjuvant chemotherapy for breast cancer (18) before the first course of chemotherapy [n = 26; 48.1 ± 8.9% (S.D.)] and before the...
sixth course \((n = 22; 43.7 \pm 12.2\%\) did not differ between each other and from the pretreatment values of the patients presented here \((n = 5; 42.2 \pm 6.5\%\) with \(p < 0.05\) in both the parametric and the nonparametric tests. Therefore, these pre-treatment data were pooled and analyzed for normal distribution by the method developed by Fisher (33). The values for skewness \((-0.4919 \pm 0.3274\) and kurtosis \((0.4771 \pm 0.6664\) were compatible with a normal distribution of the \([3H]dThd\) values. Therefore, the additional use of the Student \(t\) test on the \([3H]dThd\) data was considered to be justified.

RESULTS

**Serum Methotrexate Levels.** Serum methotrexate levels were determined 15 min, 24 hr, 48 hr, and 60 hr after completion of the methotrexate infusion (Chart 1). The 15-min values range from \(1.1 \times 10^{-4}\) to \(3.0 \times 10^{-4}\) M. These levels correspond to those reported by Stoller et al. (32) for a similar regimen. At 48 hr, all serum levels were below \(9 \times 10^{-7}\) M, the value considered to be the threshold of toxicity at that time (31). The 60-hr levels were below the limit of detection in 3 patients and below \(8 \times 10^{-8}\) M in the other 2 patients. Therefore, citrovorum factor rescue was discontinued at that time in all patients in accordance with the present concept of clinical pharmacology of methotrexate (1).

**Peripheral Blood PMN and Functional Bone Marrow Granulocyte Reserve.** As shown in Chart 2, a slight decrease of peripheral PMN occurred after HDM-CFR, reaching a nadir around Day 14. In only one instance, a value below 2000 PMN/\(\mu\)l was measured.

For technical reasons, functional bone marrow granulocyte reserve could not be serially measured in all patients. In the cases studied, no significant changes of the maximum increment of PMN after hydrocortisone were observed (Table 1).

**Peripheral Blood CFU-c.** Since the patients were in steady-state conditions with respect to their blood volume during the time of the study, the concentration of peripheral blood CFU-c can be considered as a measure of the peripheral blood CFU-c pool size (18). Immediately after HDM-CFR, the peripheral blood CFU-c pool decreased with return to pretreatment values by Day 7 (Chart 2).

**Bone Marrow Differentials.** The data of the bone marrow differentials are presented in Table 1. Before treatment, normal values for the E/G ratio and for the percentages of the morphologically identifiable granulocytic compartments were obtained (3). On Day 2 after chemotherapy, the E/G ratio was significantly decreased \((p < 0.005\), reflecting the nearly complete elimination of erythroid precursors at this time. The few erythroid cells present in marrow smears at that time showed marked morphological abnormalities: multinucleated erythroid precursors were the most striking atypia; nuclear fragments, megaloblastic changes, and irregularly shaped nuclei were other frequent findings. During early erythroid regeneration, an increase of the E/G ratio to values above normal occurred, with subsequent normalization during granulocytic regeneration.

The reduction of the erythroid compartment on Day 2 induced a relative increase of the cells of the granulocytic compartments. This relative increase was more pronounced in the maturing granulocytic compartment than in the proliferating granulocytic compartment.

**Bone Marrow CFU-c.** Data on the relative number and proliferative activity of bone marrow CFU-c are included in Table 1. Before HDM-CFR, the relative number of bone marrow CFU-c \((i.e., CFU-c/10^5\) marrow cells\) was found to be within the normal range of our laboratory (16). On Day 2, a decrease by approximately 50% occurred, with partial normalization on Day 7 and a return to pretreatment values on Day 14. The proliferative activity of bone marrow CFU-c, as measured by the \([3H]dThd\) suicide technique, increased to a moderate degree on Day 7 \((p < 0.05\) in the Mann-Whitney and in the Student \(t\) test) and returned to normal by Day 14.

**Repeated HDM-CFR and Ineffective Rescue.** The results obtained during 4 successive courses of HDM-CFR in one additional patient are shown in Chart 3. Courses 1 to 3 show essentially the characteristics described for the whole group in the previous section. Although the depression of the circulating CFU-c appeared to increase in severity from course to course, recovery occurred within an equally short time, and peripheral PMN pool, functional bone marrow granulocyte reserve, and bone marrow data were similar throughout Courses 1 to 3.
Human Granulopoiesis after High Dose of Methotrexate

Table 1
Bone marrow granulocyte reserve, CFU-c concentration, proliferative activity ([3H]dThd suicide), and bone marrow differentials during 5 initial courses of HDM-CFR

<table>
<thead>
<tr>
<th>Day</th>
<th>ΔPMN(^a)</th>
<th>CFU-c/10(^5)</th>
<th>[3H]dThd suicide (%)</th>
<th>Differential E/G ratio</th>
<th>Proliferation (%)</th>
<th>Maturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3030 ± 1060(^b)</td>
<td>37 ± 14</td>
<td>42.2 ± 6.5</td>
<td>0.78 ± 0.32</td>
<td>13.6 ± 5.1</td>
<td>28.3 ± 6.8</td>
</tr>
<tr>
<td>2</td>
<td>ND(^c)</td>
<td>23 ± 10</td>
<td>44.0(^d)</td>
<td>0.14 ± 0.08</td>
<td>18.1 ± 6.2</td>
<td>54.0 ± 12.9</td>
</tr>
<tr>
<td>7</td>
<td>2800(^d)</td>
<td>30 ± 12</td>
<td>55.6 ± 5.2</td>
<td>1.36 ± 0.31</td>
<td>12.1 ± 4.4</td>
<td>22.1 ± 3.1</td>
</tr>
<tr>
<td>14</td>
<td>5500(^d)</td>
<td>36 ± 27</td>
<td>43.0 ± 6.0</td>
<td>1.27 ± 0.64</td>
<td>12.3 ± 4.4</td>
<td>25.5 ± 13.3</td>
</tr>
</tbody>
</table>

\(^a\) ΔPMN, bone marrow granulocyte reserve.
\(^b\) Mean ± S.D.
\(^c\) ND, not done.
\(^d\) Data not complete for all courses.

In contrast to the first 3 courses, which were tolerated with only minor subjective and no objective side effects, the patient experienced severe complications during Course 4 of HDM-CFR, with marked desquamation of mucous membranes, thrombopenic bleeding, and septicemia. A continuous decline of peripheral PMN occurred, reaching a nadir of 30 PMN/μl on Day 18. Between Days 4 and 15, no CFU-c were detectable in the peripheral blood. Hematological recovery was heralded by an overshooting repletion of the peripheral blood CFU-c pool size up to a value of 6.5 × 10⁶/liter. Because of the serious condition of the patient, no bone marrow aspirations were performed during this course of HDM-CFR.

DISCUSSION

The data obtained during the first courses of high-dose methotrexate therapy with effective citrovorum factor rescue demonstrate that this chemotherapeutic regimen has remarkably little effect on granulopoiesis. Peripheral blood PMN and functional bone marrow granulocyte reserve are only marginally depressed. The decrease of the peripheral blood CFU-c pool size and of the relative number of bone marrow CFU-c is moderate, and recovery to pretreatment values occurs within 7 to 14 days.

All methods applied to characterize chemotherapy-induced changes in the human bone marrow will provide relative values. Thus, morphological studies describe the percentages of the different cell types, whereas culture data refer to the number of CFU-c per 10⁵ cultured marrow cells. Conversion of such relative data into absolute numbers reflects changes in the size of the different marrow compartments; however, this requires repeated measurements of the human marrow mass, and the methods described for this purpose (5, 8) cannot be applied repeatedly at short intervals. Therefore, any interpretation of the chemotherapy-induced pattern of depletion and regeneration of the human hematopoiesis is based on relative changes. Even with these limitations, our data are in agreement with the contention that HDM-CFR injures the human granulopoiesis rather little, when the rescue is effective.

This concept is supported by a comparison of the changes after HDM-CFR with those induced by other chemotherapeutic regimens. After cyclophosphamide-Adriamycin standard doses (18) or after high doses of cyclophosphamide (17), we have demonstrated serious damage to peripheral blood and bone marrow CFU-c and to the proliferating granulocytic compartment of the marrow. In reaction to such injury, marrow CFU-c greatly increase their proliferative activity. In contrast, the relative number of bone marrow CFU-c is only moderately reduced, and their proliferative activity is only slightly increased after successful HDM-CFR.

Since the proliferative activity of bone marrow CFU-c is not subject to the restrictions of nonquantitative bone marrow sampling, these data are of particular interest. The increase of proliferative activity, although moderate, is statistically validated at p < 0.05 by both parametric and nonparametric tests. In comparison, we have shown other therapeutic regimens to
cause significantly greater increments in proliferative activity of bone marrow CFU-c (17, 18).

Thus, quantitative and qualitative data demonstrate a limited damage not only on the morphologically identifiable granulocytic compartments but also on the CFU-c level. This is of importance, since persistent damage to the stem cell compartments despite apparent normalization of the more mature bone marrow compartments has been described in animal models. After busulfan, the CFU-c pool is markedly reduced while the proliferation and maturation compartments are found to be nearly normal (22–24, 34). Continuous irradiation causes severe depression of the stem cell compartments (35) with much less reduction of bone marrow cellularity (19).

Analysis of the morphological changes in the marrow revealed a considerable increase in the percentage of the cells of the maturing granulocytic pool shortly after HDM-CFR; this represents a concentration effect, caused by the disappearance of drug-susceptible cells, in particular of erythroid precursors. The percentage of the cells of the proliferative granulocytic pool was also increased by this time, but this relative increase was less than that seen for the maturing compartment. In contrast, the relative number of marrow CFU-c had decreased at this time. These data suggest that CFU-c are more sensitive to the cytotoxic effect of HDM-CFR than are the cells of the proliferating granulocytic pool. From our data, we cannot exclude that the relative number of marrow CFU-c declines because they differentiate into the proliferating granulocytic compartment without concomitant influx from the pluripotent stem cell pool into the CFU-c compartment. However, experimental data demonstrating influx of colony-forming unit-spleen into the CFU-c compartment in spite of continued methotrexate administration argue against this possibility (25, 26).

The almost complete disappearance of erythropoietic cells from the marrow on Day 2 after HDM-CFR points to a higher sensitivity of the human erythropoietic cells than that of the proliferating cells of the granulopoietic system. A high methotrexate sensitivity of erythropoietic cells has been reported for the rat (2), but this has not been confirmed in mice (21). The morphological changes observed in erythroid precursors after HDM-CFR resemble those after standard doses of methotrexate (30).

Following uncomplicated HDM-CFR, the moderately depleted peripheral blood CFU-c pool returned to pretreatment size without overshooting expansion. In contrast, there was a remarkable rebound of the peripheral blood CFU-c pool size following the toxic course of HDM-CFR. In our experience, this pattern of repletion of the peripheral blood CFU-c pool occurs characteristically during granulopoietic regeneration from severe cytotoxic drug-induced marrow injury (17). The present observations after toxic HDM-CFR support the previous findings after high-dose cyclophosphamide.

The present findings suggest that frequent administration of HDM-CFR at short intervals should be rather well tolerated by the human granulopoietic system, and this has indeed been demonstrated clinically (12). For clinical practice, the remarkably moderate toxicity of effective HDM-CFR on granulopoiesis should be taken into consideration where it represents an equally effective chemotherapeutic alternative to other forms of cytotoxic regimens. In the patient in whom 4 successive courses of HDM-CFR were observed, the interspersed administration of cyclophosphamide and Adriamycin did not alter the pattern of the second and third course of HDM-CFR. The reason for the toxic course during the fourth administration of HDM-CFR is not clear from our study. This patient was treated before the need for monitoring serum methotrexate levels had been generally recognized. Further studies are needed to delineate the influence of HDM-CFR on granulopoiesis of patients with suspected residual marrow damage caused by previous chemotherapy or radiotherapy in whom HDM-CFR may still be a well-tolerated and mildly toxic chemotherapeutic regimen.

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REFERENCES

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