Evidence for Early Recruitment of Granulocyte Precursors During High-Dose Methotrexate Infusions in Mice

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The effects of constant exposure to high concentrations of methotrexate in vivo on the committed stem cell (CFU-C) were studied by in vitro culture of mouse bone marrow. Bone marrow samples were obtained from animals receiving a continuous infusion, and were cultured in a methotrexate-free semisolid gel system. The effects of methotrexate infusion on the pluripotent stem cell population (CFU-S) were studied as well. Constant exposure to $10^{-5}$ M methotrexate produced a rapid decrease in total nucleated cells per femur, reaching 35% of control at 12 hr and remaining at approximately this level throughout 48 hr of drug infusion. A decrease in the number of both CFU-C and CFU-S per femur was observed, which paralleled the drop in nucleated cells during the first 24 hr. However, in contrast to an additional drop in the number of CFU-S, an increase of CFU-C number per femur was observed from 24 to 48 hr. These data indicated a self-limited cell kill of nucleated bone marrow cells, and suggested recruitment of CFU-C from the CFU-S pool between 24 and 48 hr of infusion despite continued methotrexate infusion.

Antineoplastic agents exert their primary toxic effects on rapidly dividing host tissues such as bone marrow and intestinal epithelium, in addition to their effects on malignant tissue. The relationship of various pharmacologic parameters (drug level, duration of drug exposure) to cell kill has been extensively investigated for murine and, to a lesser extent, for human neoplasms. The important effects of these agents on normal tissues are less well understood. Previous studies have established that methotrexate administration is followed by a rapid suppression of DNA synthesis in both bone marrow and gastrointestinal epithelium. A corresponding fall occurs in both the number of pluripotent stem cells (CFU-S), as measured by the spleen-colony technique, and in the number of committed granulocytic stem cells (CFU-C), as determined by in vitro culture. DNA synthesis in these tissues recovers at or below doses of $2 \times 10^{-8}$ M and exceeds pretreatment synthesis rates during the recovery phase, a change which correlates with a rapid restoration of the CFU-C pool.

The present experiments were designed to study the changes in CFU-S and CFU-C pools induced in bone marrow during constant high-dose infusion of methotrexate. A major change was observed after 48 hr infusion as compared to after 24 hr of infusion, specifically an increase in the number of CFU-C and a decrease in the CFU-S number. This change had its onset during the period of

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maximal suppression of bone marrow DNA synthesis and probably represented recruitment of CFU-C from the CFU-S pool during the presence of the drug.

MATERIALS AND METHODS

Constant blood levels of methotrexate were produced in 21 C57B1/6N male mice (16-20 g) by subcutaneous placement of constant infusion cells as described by Dedrick et al., or with osmotically driven miniature pumps (Alzet, Generic Delivery System, Alza Corp., Palo Alto, Calif.). Prior to in vivo implant, these devices were brought to equilibrium in vitro, and the MTX output of each cell was measured spectrophotometrically. Cells or minipumps were implanted into mice anesthetized with ether. Sufficient devices were used per mouse to give a total output between 100–200 µg/hr at 37°C as determined in vitro. Simultaneously, each mouse received a pulse dose of methotrexate (5 mg/kg intraperitoneally) in order to block dihydrofolate reductase activity and produce more rapid equilibrium at the desired plasma concentrations. Blood samples were taken from the retro-orbital sinus of control and experimental animals at 2, 5, 10, and 24 hr in the 24 hr experiments, and also at 36 and 48 hr in the 48 hr experiments. Methotrexate was assayed by enzyme inhibition and enzyme competitive binding methods.

The mice were killed by cervical dislocation after 12, 24, or 48 hr of infusion. The infusion devices were removed and rechecked for in vitro methotrexate output.

Bone marrow of each mouse was cultured in quadruplicate. Preparation of the marrow suspension, methylcellulose culture medium, plating technique, colony counting, and calculations were done as previously described. L-cell supernatant was used as a colony-stimulating factor. The marrow was washed three times in McCoy's medium prior to plating in order to eliminate any extracellular methotrexate.

In general, 7.5 x 10^4 nucleated cells were added per plate and cultures were incubated at 37°C under 7%-10% CO₂ for 7-10 days, at which time the number of colonies containing 50 cells or more was determined. Infusion devices lacking methotrexate were inserted in eight additional mice in order to determine the effect of the glass micropipettes on colony formation, and no effects were seen in comparison to controls not subjected to surgery.

The spleen colony assay for CFU-S was kindly performed by Dr. Allan Wu of Litton-Bionetics, Bethesda, Md., according to the procedures of Till and McCulloch. Irradiation of mice was done with a Westinghouse Constant Potential x-ray machine (with 250 KV) at NIH, Bethesda, Md. The dose of irradiation was first calibrated to eliminate endogenous colony formation and to maintain a good survival of irradiated mice. C57BI/6N mice, receiving cell suspensions at 8 wk of age, were first given 550 rads 1 hr before intravenous injection of the cells. Pooled bone marrow cells from three mice infused with methotrexate for 24 hr, from three mice infused for 48 hr, and from three control mice were used; 5 x 10^4 cells of the three cell suspensions were each injected into six to eight recipient mice. The recipient mice were killed 10 days later, and their spleens were removed and placed in Bouin's fixative; the number of macroscopic colonies on the surface of each
spleen was then counted. The mean number of colonies in each of the three groups was calculated and expressed as the total number of CFU-S per femur.

The effects of methotrexate infusion on DNA synthesis in mouse bone marrow in vivo were determined by the incorporation of $^{3}H$-deoxyuridine ($^{3}H$-UdR) into DNA according to previously described methods.\textsuperscript{10}

RESULTS

A plateau in the plasma concentration of methotrexate was reached 2.5–5 hr after insertion of the infusion devices, and only minor variations were observed thereafter during the infusion (Fig. 1). The effects of these constant plasma concentrations in the range of $0.5–2.0 \times 10^{-5} M$ on the total number of nucleated cells and CFU-C is shown in Fig. 2. The total number of nucleated cells per femur fell to a nadir of 35% of control by 12 hr and remained in the range of 25%–35% of control throughout the remainder of the 48-hr infusion. During the first 24 hr of drug infusion, the percentage of CFU-C per $7.5 \times 10^{4}$ nucleated cells remained constant and the total number of CFU-C per femur declined parallel to the fall in total nucleated cells. However, during the period from 24 to 48 hr the percentage of CFU-C increased in all five animals studied (Fig. 2) as compared to control mice ($p < 0.002$) and as compared to marrow from animals receiving 12 or 24 hr infusions ($p < 0.001$). The number of CFU-C per femur was calculated from these data and was significantly increased at 48 hr as compared to 24 hr ($p < 0.03$). The increase in CFU-C during the second 24 hr of infusion occurred despite a further minor decrease in the total number of nucleated cells per femur.
The percentage of polymorphonuclear cells and monocytes is known to influence the plating efficiency of CFU-C.\textsuperscript{15,16} However, comparison of smears of control, 24-hr, and 48-hr bone marrow specimens revealed no change in the relative numbers of these cells.

To rule out the possibility that culture of $7.5 \times 10^4$ cells was resulting in a disproportionately high number of colonies in the experimental group, a titration curve was performed in the range of $2.5-10 \times 10^4$ cells per plate. Figure 3 shows that there was a linear relationship of inoculum size to CFU-C for both experimental and control marrow cells and a consistently increased percentage of CFU-Cs in the experimental animals at all inoculum levels.

The increase in CFU-C between 24 hr and 48 hr suggested recruitment of myeloid precursors from a pool of more primitive cells, such as those measurable in the CFU-S assay. Therefore, we determined the change in the number of CFU-S per $10^5$ cells and per femur during methotrexate infusion. A 63% decrease in the total number of CFU-S was observed during the 0–24-hr period of infusion, and a further decline to 12% was seen during the 24–48 hr period (Table 1). This decline in CFU-S in the second 24-hr period was comparatively greater than the fall in total nucleated cells, and was reflected in the fall in CFU-S/$10^5$ cells from 40 at 24 hr to 22 at 48 hr. This fall in CFU-S occurred during the interval when the total number of CFU-C was increasing and supported the hypothesis that CFU-C were being recruited from the CFU-S pool during this period.

Previous experiments have shown a spontaneous resumption of DNA synthesis in bone marrow during continuous exposure to $2 \times 10^{-8} M$ methotrexate

| Table 1. Decrease in Spleen Colony-forming Cells During Methotrexate Infusion |
|-----------------|-----------------|-----------------|
| Total Nucleated Cells | CFU-S/10^5 Cells | CFU-S/Femur (%) Control |
| Control          | 100             | 42              | 8823 (100)               |
| After 24 hr infusion | 39              | 40              | 3280 (37)                |
| After 48 hr infusion | 23              | 22              | 1056 (12)                |
in mice.\textsuperscript{10} In order to determine whether a similar resumption of DNA synthesis accompanied the increase in CFU-C between 24 and 48 hr of high-dose methotrexate infusion, the incorporation of \textsuperscript{3}H-UdR into DNA of bone marrow was determined in vivo. \textsuperscript{3}H-UdR incorporation fell within 1 hr to less than 5\% of control values, and continued suppression was seen throughout the 48 hr infusion, indicating a continuous block in DNA synthesis by methotrexate. Thus, at the time of increased colony-forming potential as measured in vitro, no increase in DNA synthesis was seen in vivo. However, removal of the infusion cells at 48 hr resulted in a rapid increase in \textsuperscript{3}H-UdR incorporation at 72 and 96 hr to greater than control levels.

**DISCUSSION**

Although the myelosuppressive toxicity of methotrexate has been recognized since the drug's first use, there exists at present only a limited understanding of the pharmacologic parameters and host responses which determine the magnitude of this toxicity. The development of techniques for bone marrow culture in vitro, for measuring spleen colonies in vivo, and the design of constant infusion devices for drug administration in vivo have allowed studies of the sequence of effects of methotrexate on mouse bone marrow exposed to constant drug levels, and have delineated the following events. A rapid suppression of DNA synthesis is observed, and correlates with a significant decrease in the number of nucleated cells and CFU-C per femur during the first 12 hr. Thereafter, little further fall in nucleated cells per femur is observed despite continued drug infusion, probably due to the self-limiting effects of methotrexate on protein and RNA synthesis, which prevent progression of cells into the vulnerable DNA synthetic phase of the cell cycle.\textsuperscript{17}

Although methotrexate levels of $10^{-5} \text{M}$ maintained a continuous inhibition of DNA synthesis, important changes were observed in bone marrow colony-forming ability during the infusion period. A significant increase in the percentage and total number of CFU-C occurred between 24 and 48 hr as compared to the number at 24 hr. This change was probably the result of recruitment of CFU-C by differentiation from a pool of more primitive cells, such as the CFU-S, in response to the cytotoxic agent.

Additional experiments showed that the increase in CFU-C could not be attributed to (1) a change in the percentage of granulocytes or monocytes in the marrow populations, or (2) a change in titration curve relating the number of cells plated to the CFU-C colonies grown (Fig. 3). It is of interest that the increase in CFU-C occurred during the infusion period despite the continuous suppression of DNA synthesis. Recovery of DNA synthesis was seen only after elimination by the washing procedure in vitro, or by discontinuation of the infusion in vivo.

Data from previous studies\textsuperscript{18} show that the number of CFU-S drops to about 30\% of control during the first 12 hr of multiple daily doses of methotrexate and remains at this nadir throughout a 72-hr period of drug administration. Our findings with continuous high dose infusion show a further drop in CFU-S during the second 24-hr period, coinciding with the rise in CFU-C. The observations in the present study are consistent with the hypothesis that recruit-
ment of CFU-C occurs from the CFU-S pool during the high-dose infusion, although selective killing of CFU-S during this period could be an alternative explanation for this finding. A second possible explanation for the disproportionate fall in CFU-S at 48 hr could be a selective migration of these cells to other body compartments. If the interpretation is correct that CFU-C are being recruited from the CFU-S pool during drug infusion, then this finding implies that progression of cells from the CFU-S pool to the CFU-C pool of myeloid precursors does not require a cell division step.

Similar experiments in man might be of value in order to determine whether a brief period of cell kill, followed by blocking of cell cycle progression and recruitment of myeloid precursors also occurs in humans. Comparable bone marrow studies during high-dose methotrexate infusion would have a considerable impact on determining the proper duration and sequence of therapy with methotrexate and other cytostatic drugs.

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REFERENCES

