The Relative Contribution of Drug Concentration and Duration of Exposure to Mouse Bone Marrow Toxicity during Continuous Methotrexate Infusion

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SUMMARY

The effects of exposure of bone marrow to specific methotrexate (MTX) concentrations were studied by constant infusion of the drug into C57BL mice. The residual marrow nucleated cell count was determined in 168 mice at specific intervals. In vitro culture of colony-forming cells (CFU-C) was also performed in 69 of these mice. Duration of exposure varied from 12 to 72 hr. Plateau plasma MTX concentrations were studied in the range from $10^{-8}$ to $10^{-5}$ M. The total number of nucleated cells per femur fell to a nadir in vivo during exposure to $10^{-6}$ M MTX. The percentage of CFU-C per $7.5 \times 10^6$ nucleated cells plated increased after 48-hr infusions compared to the percentage after 24-hr infusions. This increase was seen at all plasma concentrations studied. The total number of CFU-C per femur at plasma MTX concentrations above $10^{-6}$ M decreased in the first 24 hr to 40% of control, but then the number significantly increased to 66% of control between 24 and 48 hr. In contrast, no change was observed in CFU-C per femur between 24 and 48 hr during constant infusion at plasma concentrations below $10^{-6}$ M. Wright's-stained smears showed no change in the differential count of marrow specimens at 24 and 48 hr that might account for the increased percentage of CFU-C at 48 hr. The increase in CFU-C per femur during high-dose infusions is probably the result of recruitment of CFU-C. The increased percentage of CFU-C suggests recruitment at the lower concentrations as well, but selective elimination of non-CFU-C cells cannot be excluded. Marrow [6-³H]deoxyuridine incorporation studies in vivo during exposure to $10^{-7}$ M MTX showed that the phenomenon of recruitment observed in vitro was initiated during the absence of DNA synthesis in vivo.

INTRODUCTION

MTX, a folic acid analog with potent antineoplastic activity, is used in a variety of clinical schedules and combination therapy regimens in man (1, 8, 11). Although the pharmacokinetics of MTX has been investigated in detail in both man (20) and animals (3), the relationship of drug concentration and duration of tissue exposure to cell kill is still poorly understood. Previous studies in which single-bolus doses of MTX (3) were used to study toxicity to normal tissues, particularly to bone marrow (4, 6, 23) and intestinal mucosa (13), have shown that plasma MTX concentrations above $1 \times 10^{-6}$ M are associated with inhibition of DNA synthesis in bone marrow, whereas the threshold for inhibition of intestinal mucosal DNA synthesis appears to be somewhat lower, approximately $5 \times 10^{-6}$ M. Constant infusion of MTX to achieve a plasma level of $2 \times 10^{-6}$ M revealed an initial suppression of [³H]UdR incorporation into DNA of marrow, followed by a spontaneous recovery of [³H]UdR incorporation despite continued exposure to the drug (25). Using the spleen colony assay for marrow colony-forming units (CFU-S), Bruce et al. demonstrated (5, 6) an initial rapid decrease of CFU-S after multiple doses of MTX with no further decline after 12-hr exposure. In none of these studies was it possible to quantify the relationship between drug concentrations, duration of exposure, and marrow cell kill.

More recently, using constant-infusion devices in mice, we obtained evidence for the following sequence of changes in bone marrow colony-forming cells during continuous exposure to $10^{-6}$ M MTX; (a) rapid depression of deoxyuridine incorporation into DNA and depletion of nucleated cells in the marrow, reaching a maximum within 24 hr; (b) recruitment of new myeloid CFU-C despite continued drug infusion and progressive decrease in nucleated cells; and (c) rapid resumption of DNA synthesis in surviving cells upon removal of the drug (15, 16).

These findings prompted further investigation of the effects of drug level and duration of exposure on marrow cytotoxicity and clonogenic capacity over a wide range of plasma MTX concentrations (from $10^{-6}$ to $2 \times 10^{-5}$ M) maintained for periods up to 72 hr. The results indicate that the degree of bone marrow toxicity is a function of both drug concentration and duration of exposure during the initial period of rapid cell kill. Eventually, there is a common plateau of residual nucleated cells for all drug concentrations tested. In addition, the time course of adaptive response in marrow is also a function of concentration and duration of exposure.
MATERIALS AND METHODS

General Plan of Experiments. All experiments were done with 6- to 8-week-old male C57BL mice, weighing 16 to 18 g. The 168 mice studied were exposed to a constant concentration of MTX for 12, 24, 48, or 72 hr. At the end of exposure time, the mice were killed; the TNC/f was determined for each mouse; and bone marrow colony formation (CFU-C) was determined individually by in vitro culture for 69 mice at 24 and 48 hr after infusion. In addition, in each experiment 1 mouse received no drug and served as a control. Smears of 24- and 48-hr marrows were compared microscopically with those of control marrows for differential count.

Blood samples and bone marrow were collected at identical intervals in both the control and experimental mice to correct for the effect of experimental manipulations on bone marrow colony formation. Plasma MTX concentrations were determined by a competitive binding assay (14) and verified by an enzymatic assay (2). Mean plateau values calculated for various infusion rates represent the average of 2 to 4 samples taken at 12- to 24-hr intervals during infusion. For determination of the relationship between drug concentration and colony-forming potential, the mice were grouped according to plateau plasma MTX concentrations at the following ranges: Group A, 1 to 8 x 10^-8 M (mean, 4.5 x 10^-8 M; CV, 54%); Group B, 8 x 10^-8 to 4 x 10^-7 M (mean, 1.8 x 10^-7 M; CV, 49%); Group C, 4 x 10^-7 to 2 x 10^-6 M (mean, 1.2 x 10^-6 M; CV, 44%); and Group D, 2 x 10^-6 to 2 x 10^-5 M (mean, 7 x 10^-6 M; CV, 48%). For clarity the experiments are also indicated by the duration in hr of drug infusion; e.g., A24 represents 24-hr infusion for Group A.

Drug Administration and Infusion Devices. A priming MTX dose of 5 mg/kg i.p. was given to all mice to induce immediate inhibition of dihydrofolate reductase. This inhibition persisted over the 5- to 8-hr period (Chart 1) that is required for the MTX infusion to produce a plateau level of the drug in the plasma.

Desired blood levels of MTX were achieved and maintained with special cells, as described elsewhere (10, 17). Briefly, these special cells were glass micropipets (5 to 200 µl) from which MTX diffuses through an agar gel. Pipets with varying agar lengths were used to obtain different outputs of the drug. Cell output was verified in vitro by measuring MTX concentration spectrophotometrically at 302 nm. The infusion devices were then placed s.c. in mice that were lightly anesthetized with ether. For plasma concentrations of the drug above 5 x 10^-7 M, multiple cells were required. In a few experiments, osmotically driven miniature pumps (ALZET; Generic Delivery System, Alza Corp., Palo Alto, Calif.) were used to obtain a plasma MTX concentration of 10^-8 M. These pumps were cylindrical, had a diameter of 0.6 cm and a length of 2.5 cm, and contained 150 µl of MTX solution, which was delivered at a rate of 300 µg/hr in vivo. After the infusion devices were removed from the mice, the constancy of in vitro output was redetermined.

Preparation and Culture of Bone Marrow. After the mice were killed, both femurs were aseptically removed. The marrow was expelled from the medullary cavity into 2 ml of McCoy's medium, as described elsewhere (4). Nucleated cells were counted with a hemocytometer; and the count was converted to TNC/f. The cells were washed and centrifuged 3 times at 4° in cold McCoy's medium to remove extracellular MTX. The absence of MTX was verified by assaying these wash fluids. The 1st wash fluid contained MTX at concentrations between 10^-8 and 10^-7 M; the precise level depends somewhat on the plasma concentrations to which the marrow had been exposed. After 2 washes the concentration drops below 10^-6 M in the fluid of subsequent washes. At this concentration the cloning capacity of marrow cells is not sensitive to MTX.

These washed cells were then grown to clones in semi-solid methylcellulose according to the technique of Worton et al. (24) with minor modifications (4, 16). L-cell supernatant was used as colony-stimulating factor. The experiments were done in quadruplicate with plating of 7.5 x 10^4 nucleated cells per Petri dish.

After 7 days of incubation, the total number of colonies containing 50 or more cells (range, 50 to >5000) was counted on each plate. The percentages of CFU-C and CFU-C/f were calculated from these data (4).

The CFU-C cultured in this system are defined as stem cells committed to granulopoiesis. In contrast, the CFU-S are uncommitted stem cells that will not form clones in vitro but will form clones in vivo in the spleens of irradiated mice (6).

Effect of MTX on [³H]UdR Incorporation in Vivo. In a separate group of 16 mice, [³H]UdR (5 µCi and 0.4 µg per g mouse weight) was injected via a tail vein at chosen intervals during MTX infusion. The mice were killed 20 min later;
Bone Marrow Toxicity during MTX Infusion

Bone marrow cells were removed and freed of fat and connective tissue; and marrow cells were frozen with liquid nitrogen. These studies were done at 5, 7, 17, 25, 29, and 48 hr on groups of 2 mice each exposed to an MTX plasma level of $2 \times 10^{-2} \text{ M}$. We also studied [3H]UdR incorporation into DNA in 4 mice at 72 and 96 hr after termination, at 48 hr, of MTX infusion. All tissues were kept at $-15^\circ$ until analysis. DNA was extracted and measured according to the method of Schneider (19) as modified by Burton (7); tritium in the extract was measured by scintillation counting.

RESULTS

MTX Plasma Concentration in Relation to Dose. A linear relationship was found between the rate of MTX output by infusion cells and the plasma plateau concentration of MTX. A dose of $0.2 \mu\text{g/hr}$ gave a plasma concentration of about $10^{-6} \text{ M}$, whereas a dose of $200 \mu\text{g/hr}$ resulted in a 3-log increase in the level to approximately $10^{-4} \text{ M}$. Although the dose-concentration relationship was linear when the dose was increased from 0.2 to 100 $\mu\text{g/hr}$, levels achieved with infusions greater than 100 $\mu\text{g/hr}$ slightly exceeded the expected plasma level; complete data are presented in a separate publication (17).

Chart 1 gives an indication of the plasma concentrations actually achieved in some mice in these experiments. For comparison, the dashed line gives the plasma concentration that would result from a single 5-mg/kg i.p. dose (9).

Total Nucleated Cell Depletion. The fall in the TNC/f after exposure to various plateau concentrations of MTX for 3 different periods (24, 48, and 72 hr) is shown in Chart 2. With all concentrations of MTX above $10^{-6} \text{ M}$, TNC/f declined, reaching a nadir of approximately 30% of the control. This nadir was reached more rapidly at higher concentrations of MTX, as shown schematically in Chart 3. At $2 \times 10^{-3} \text{ M}$ MTX the nadir was reached at 12 hr of exposure, whereas at $5 \times 10^{-4} \text{ M}$ the maximum depletion was not reached until 72 hr of exposure. On reaching the maximum depletion of the TNC/f, which was similar for all concentrations of MTX, little further change in cell numbers was seen despite continued drug infusion, which probably indicates a block in cell cycle progression with limited entry of cells into the vulnerable DNA synthesis period. Smears of marrows exposed for 24 and 48 hr showed the same differential counts as did those of the controls.

CFU-C/7.5 $\times 10^2$ Plated Cells. The relationship of plasma MTX to the number of colonies per 7.5 $\times 10^2$ plated cells of bone marrow from mice exposed to the drug for 24 and 48 hr is shown in Chart 4. After 24 hr of infusion, there is no significant change in the percentage of CFU-C. There appears to be a slight, although statistically insignificant, increase in the percentage of CFU-C over that of controls as the plateau plasma MTX concentration increases. In contrast, a statistically significant increase above that of controls in the percentage of CFU-C is seen at all MTX concentrations maintained for 48 hr.

CFU-C/f. The absolute number of CFU-C/f is a more important indication of drug effect on the regenerative capacity of bone marrow than is the percentage of CFU-C (Chart 5). As MTX concentration is increased, a decrease to a plateau of CFU-C/f is seen after 24 hr of infusion. Recall

\[ \text{S.E.M. Controls} \]

\[ \text{F.S. Surviving Nucleated Cells} \]

\[ \text{Plasma MTX Molarity} \]

\[ 1 \times 10^{-8} \quad 1 \times 10^{-7} \quad 1 \times 10^{-6} \quad 1 \times 10^{-5} \]

\[ \text{FEBRUARY 1977} \]
from Chart 2 that this decrease is paralleled by a decrease in TNC/f. However, after 48 hr of infusion CFU-C/f did not differ significantly from the 24-hr values until the highest drug concentration of 48 hr (D48) was reached. Compared with the 24 hr (D24) value, there is a significant difference despite continued drug infusion with no change in TNC/f (Chart 2). This finding suggests that recruitment takes place in response to the cytotoxic effect of the prolonged drug infusion at the highest concentration.

Effect of Constant Infusion of MTX on [3H]UdR Incorporation into DNA. This portion of the study was performed in mice with plateau plasma concentrations of $2 \times 10^{-8}$ M (Group A) and $2 \times 10^{-7}$ M (Group B); Chart 6 shows the effect on bone marrow of these plasma concentrations. The inhibition of [3H]UdR incorporation is least rapid at $2 \times 10^{-8}$ M and reaches a minimum of 22% at 12 hr. A spontaneous recovery of [3H]UdR incorporation is observed between 24 and 48 hr despite the continued presence of $2 \times 10^{-8}$ M MTX, whereas steady inhibition is maintained over a 48-hr period at $2 \times 10^{-7}$ M plasma MTX. Upon termination of the infusion by removal of the constant-infusion device, a rapid recovery is seen in Group B (plasma level, $2 \times 10^{-7}$ M) with an overshoot of [3H]UdR incorporation at 72 and 96 hr. Similarly, the recovery that begins prior to 49 hr during drug infusion at $2 \times 10^{-8}$ M shows a further increase (to approximately 300%) of the control value after removal of the infusion cell at 48 hr.

Sensitivity of CFU-C to MTX after in Vivo Exposure to MTX for 48 Hr. To investigate the mechanism underlying the spontaneous resumption of DNA synthesis during the infusion of $2 \times 10^{-8}$ M MTX (25), additional experiments were performed. Bone marrow was removed from mice infused...
with MTX at $2 \times 10^{-8}$ M for 48 hr and was cultured in vitro with MTX in concentrations ranging from $1 \times 10^{-8}$ to $5 \times 10^{-7}$ M in a medium containing dialyzed fetal calf serum (Chart 7). MTX sensitivity was similarly evaluated in marrow from control animals that received no MTX. Inoculation of each plate with $7.5 \times 10^{9}$ nucleated cells yielded 55 colonies for control marrow and 85 colonies for marrow from animals exposed to $2 \times 10^{-6}$ M MTX. In the presence of $1 \times 10^{-8}$ M MTX, the number of colonies was reduced by 50%, for both control and experimental marrows, which indicates the equal sensitivity of these cells to inhibition by MTX. Thus, spontaneous resumption of DNA synthesis during infusion of $2 \times 10^{-8}$ M MTX appears not to be the result of the selection of cells that are less sensitive to MTX. Our hypothesis that there is recruitment of additional cells for colony-forming activity with unchanged sensitivity to MTX is further supported. This recruitment could account at least partially for the apparent increase in [$^{3}$H]UdR incorporation per $\mu$g DNA during the 2nd 24 hr of MTX infusion; i.e., from 30 to 100% of control.

**DISCUSSION**

The present investigation was made possible with constant-infusion devices, which were shown to produce predictable plasma levels based on their in vitro output of drug. An approximately linear relationship between rate of drug output and plasma level is observed. Previous clinical studies have suggested that toxicity to MTX is more a function of the duration of drug administration than a function of the dose of the drug (21). With plasma concentrations maintained at the chosen levels by constant infusions, we were able to relate the bone marrow toxicity to specific concentrations of MTX present for a specific period of time. We have reported the plasma concentrations as total concentration. In those species that have a significantly different degree of plasma protein binding than have mice (approximately 20%), appropriate adjustments will have to be made if extrapolation is attempted.

During constant infusion of MTX, a maximum depletion of 70% of nucleated cells from bone marrow was observed for all concentrations between $1 \times 10^{-8}$ and $1 \times 10^{-6}$ M. Maximum depletion was reached more rapidly at higher drug levels, which can be seen in Charts 2 and 3. For a given concentration of MTX, the fraction of surviving nucleated cells was a function of duration of exposure until a plateau was reached. Thus, despite virtually complete inhibition of [$^{3}$H]UdR incorporation into DNA at all drug levels above $1 \times 10^{-7}$ M, a more rapid cell depletion was observed at the higher plasma concentrations.

During the period of drug infusion, the changes in numbers of in vitro colony-forming marrow cells initially paralleled that of the total nucleated cells. However, an increasing percentage of CFU-C per fixed number of plated cells was seen during continued infusion from 24 to 48 hr. Thus, an absolute increase in CFU-C/f was seen in the period from 24 to 48 only at the highest plasma level. Although this increase in percentage might be explained as a more selective cytotoxic effect of MTX on those nonclonogenic nucleated cells, this explanation is unlikely because the increase CFU-C/f occurred although the TNC/f remained constant between 24 and 48 hr. Further evidence that recruitment is actually occurring is provided by our recent finding (16) that there is a progressive drop in CFU-S during the interval from 24 to 48 hr of high-dose MTX infusion. Our suggestion is that the CFU-C are "recruited" from the CFU-S pool thus decreasing the size of this pool.

The increased percentage of CFU-C and the spontaneous resumption of DNA synthesis during low-dose infusions suggest that this recruitment takes place even at low-MTX infusions. Experimentally, this recruitment could be statistically shown only with CFU-C/f during the high-dose infusions in which the decrease in TNC/f had reached a plateau.

Recruitment of CFU-C during infusion may be responsible for the marked overshoot in DNA synthesis observed after removal of the infusion cells. At low plasma levels of $2 \times 10^{-8}$ M, an increase in [$^{3}$H]UdR incorporation into DNA was observed at 48 hr despite continued drug infusion. The marrow that accounted for this increase proved to harbor CFU-C with the same sensitivity to MTX as control marrows had. Thus, the spontaneous increase in DNA synthesis observed after 24 hr at $2 \times 10^{-8}$ M is not a result of induced cellular resistance to MTX but may be a result of recruitment. A small percentage of these cells are able to enter the S phase in the presence of $2 \times 10^{-6}$ M MTX (0.8 $\mu$g/hr infusion [Chart 6]). However, at the higher concentration of MTX of $2 \times 10^{-7}$ M (10 $\mu$g/hr infusion), even the recruited cells are likely to be blocked in vivo, as indicated by in vitro tests in Chart 7, and thus show no spontaneous recovery of DNA synthesis in vivo. It is not until these cells are removed from the mice, washed free of MTX, and cloned in vitro that this recruitment can be detected.

The increase in size of individual colonies of marrow cells exposed to the highest concentrations of the drug during 48 hr can be explained by an earlier observation that colony size is a function of the number of colonies per plate (18). Thus, the increase in size results from some stimulation on the plate provided by an increased number of colonies.

These studies performed during continuous drug infusion
differ somewhat from previous studies in which multiple intermittent injections of the drug were used. Bruce et al. (5) administered MTX, 10 mg/kg, every 4 hr. His data show a nadir in the number of CFU-S, which was reached at 12 hr and persisted throughout a 48-hr period of treatment. The interpretation of these data in light of our findings is clouded somewhat by the wide fluctuations in plasma MTX concentrations produced by this schedule of repeated single injections.

Increased toxicity can be anticipated when either drug concentration or the duration of infusion is increased in the high-dose MTX infusion protocols currently used in adjuvant therapy for osteogenic sarcoma (12). The recruitment of bone marrow CFU-C demonstrated in this study indicates the need for extreme caution in the timing of a 2nd dose of MTX or of any other S-phase-specific agent after continuous exposure to MTX. Such close scheduling of drugs may lead to the killing of a large number of recruited CFU-C and to a large reduction in the number of CFU-S, which could result in a prolonged period of aplasia. Such an effect has been demonstrated with hydroxyurea on the bone marrow of mice (22).

REFERENCES

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