Role of Drug Concentration, Duration of Exposure, and Endogenous Metabolites in Determining Methotrexate Cytotoxicity

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SUMMARY

The cytotoxic effects of methotrexate for normal and neoplastic tissues are well known to clinicians but the scheduling of drug administration has primarily been based on empiric observation. However, experimental studies in recent years have provided a rational basis for understanding the role of specific drug concentrations, durations of exposure, and physiologic environment in determining the cytotoxicity of methotrexate. The studies reviewed in this paper demonstrate the requirement for free drug in excess of approximately $10^{-8}$ M concentration for inhibiting DNA synthesis in mouse bone marrow in vivo and in vitro, and in various cell culture lines in vitro. Rapid reversal of this inhibition is possible by removal of free drug by washing of cells, enzymatic cleavage of drug, or removal of infusion source in vivo. This requirement for free drug is thought to be the result of the incomplete titration of dihydrofolate reductase by stoichiometric amounts of drug, as demonstrable in in vitro competitive binding experiments. Further studies with constant drug infusion in mice have delineated the relative importance of duration of exposure and drug concentration in determining bone marrow toxicity. It was shown that for drug concentrations $>10^{-4}$ M, the rate of depletion of nucleated bone marrow cells was directly related to plasma methotrexate concentration and reached its nadir of 30% of control within 12 hours at $10^{-5}$ M MTX, 24 hours at $2 \times 10^{-4}$ M MTX, and 48 hours at $2 \times 10^{-3}$ M MTX. The rate of loss of nucleated cells was directly related to duration of exposure until the nadir in cell count was reached. Depletion of total nucleated cells was mitigated somewhat by probable recruitment of previously uncommitted precursor cells to myeloid colony-forming potential during the second 24 hours of drug infusion. It was also found that 10% fetal calf serum as well as thymidine ($10^{-3}$ M) plus a purine nucleoside could prevent the cytotoxic effect of methotrexate on bone marrow colony formation in vitro, indicating that endogenous nucleosides may be important in determining cell-kill.

[Cancer Treat Rep 61:709-715, 1977]
gross parameters such as organ toxicity and clinical antitumor effect as the major guidelines. The only clear exception to this clouded picture is the developing knowledge of the clinical pharmacology of methotrexate and, specifically, the relationship between cytotoxicity and the concentration of free drug in plasma (5) and cerebrospinal fluid (6). It will be the purpose of this paper to summarize recent work which has established (a) the requirement for free drug in excess of the tightly bound fraction for inhibition of DNA synthesis; (b) the threshold concentration of free drug for inhibition of DNA synthesis in target organs; (c) the relationship of drug level and duration of exposure to cytotoxicity at concentrations above the threshold; and (d) the possible influence of endogenous factors such as competitive substrate, thymidine, and purine nucleosides on the cytotoxic effects of methotrexate on mouse bone marrow. Although very little of this work has been performed in man, the correlations observed undoubtedly hold true in a qualitative sense, and with the development of improved assay techniques and renewed interest in methotrexate (7,8), confirmation of these findings in man is to be expected in the near future.

The Role of Free Methotrexate

Methotrexate is a strong inhibitor of bacterial and mammalian dihydrofolate reductases, displaying stoichiometric binding to the mammalian enzyme under conditions of slightly acid pH (6,1) and limited competitive substrate (9). The \( K \) for this inhibition has not been precisely determined by conventional enzyme-kinetic methods because of technical problems associated with measuring enzyme rates under the conditions required, although estimates in the range of \( 10^{-10} \) to \( 10^{-11} \) M have been made (10). Using competitive protein-binding methods, Myers et al (8) were able to determine a \( K \), of \( 5 \times 10^{-9} \) M for the \textit{Lactobacillus casei} reductase, and a similar figure for enzyme from the human lymphoblastoid line, CEM.\(^1\) In these competitive binding experiments, as well as in conventional enzyme kinetics, it was apparent that in order to completely saturate the ligand protein, a considerable excess of free drug was required (fig 1). In considering the more alkaline pH of the intracellular milieu and the increase in competitive substrate, dihydrofolate (11), which takes place following blockade of reductase, it would be expected that a considerable excess of "free" methotrexate would be required to saturate the enzyme.

\(^1\) Myers CE. Personal communication.

These theoretic considerations correlate well with the observed requirements for methotrexate cytotoxicity in cell culture systems and in vivo. Goldman (12), using mouse L cells in culture, showed reversal of inhibition of DNA synthesis by removal of extracellular drug, despite prior apparent saturation of "high affinity sites." Residual free methotrexate was clearly inhibitory at levels of \( \geq 0.12 \mu M \), and there appeared to be correspondingly greater inhibition with higher levels of free drug. That a requirement for free drug existed in vivo was suggested by experiments in which a folate-cleaving enzyme, carboxypeptidase G\(_2\), was able to rescue mice by hydrolysis of residual extracellular drug given 24 hours after administration of an otherwise lethal dose of methotrexate (13). These experiments indicated the importance of two factors, free extracellular drug and, by inference, the duration of tissue
exposure, in determining the toxicity of this agent. More recent work by Sirotnak and Donsbach (14) has shown that intracellular levels of methotrexate at least equivalent to dihydrofolate reductase levels were required to detect partial inhibition of DNA synthesis in L1210 cells in vivo; they found that the in vivo requirement of $10^{-6}$ M methotrexate for 50% inhibition of deoxyuridine incorporation was considerably higher than needed in vivo to produce equivalent effects. In intestinal mucosa, they again found that drug levels exceeding the concentration of enzyme were required for maximal inhibition of DNA synthesis, and attributed this requirement to the nonstochiometric titration of enzyme by inhibitor (15).

This experimental work suggested that extracellular drug levels, which are in rapid exchange with unbound intracellular drug, might be monitored as an indicator of free intracellular drug, and thus might have a useful relationship to intracellular drug effect. While the relationship between extracellular and intracellular drug concentration has been determined for several experimental tumors (16) and for intestinal mucosa (15), it is still to be worked out for most tissues.

Initial efforts have been directed at establishing the lowest toxic level of free extracellular drug, a level which would correspond to the minimum inhibitory concentration used in bacteriology. While tissue culture experiments suggested that levels as high as $10^{-6}$ M were required to inhibit DNA synthesis (12), the lower concentration ($10^{-8}$ M) appears to be the threshold required for suppression of DNA synthesis in vivo in several tissues (17) including mouse and human bone marrow (fig 2). This conclusion was reached in pulse-dose experiments which correlated plasma levels of methotrexate with the incorporation of $^3$H-deoxyuridine into DNA in the target tissue. Somewhat lower concentrations ($5 \times 10^{-9}$ M) were required to inhibit DNA synthesis in gastrointestinal mucosa. Constant infusion devices were used by Zaharko and co-workers (18) to set plasma methotrexate at specific levels, and these experiments provided unambiguous confirmation of partial inhibition of DNA synthesis in bone marrow at levels of $2 \times 10^{-9}$ M, and a more complete inhibition of intestinal mucosa at this level. An important common finding of the foregoing studies and the reports of Sirotnak and Donsbach (14,15) was that the target tissues (bone marrow, intestinal mucosa, and various tumor cells) varied in their sensitivity to specific extracellular concentrations of methotrexate. Zaharko et al (18) suggested that these differences in sensitivity could be attributed
to differences in the "permeability" of the tissues to methotrexate. Sirotnak clearly demonstrated differences in the persistence of free methotrexate in the more sensitive L1210 cells compared to intestinal mucosa after pulse doses of drug. As yet, the reason for differential drug persistence in the various target tissues is not understood, although most investigators agree that differences in the transport mechanisms of these tissues must be responsible. While the experiments in whole animals in general agree with the results in tissue culture, the requirement for high concentration of methotrexate (10^{-8} M) to inhibit DNA synthesis in mouse L cells in tissue culture has prompted Goldman (12) to postulate the existence of a second "low affinity" site of dihydrofolate reduction.

There are some difficulties in accepting a strictly quantitative interpretation of the foregoing studies, all of which have utilized 3H-deoxyuridine incorporation into DNA as the basis for assessing the inhibitory effects of methotrexate on DNA synthesis. Agents such as methotrexate, 5-fluorouracil, and 5-fluorodeoxyuridine which inhibit the synthesis of thymidylate, lead to marked expansion of the deoxyuridine-5'-monophosphate (dUMP) pool, into which the labeled nucleoside, 3H-deoxyuridine, must be diluted before conversion to deoxythymidine-5'-monophosphate (dTMP) and incorporation into DNA. Expansion of the dUMP pool was monitored following 5-fluorouracil and 3H-deoxyuridine incorporation corrected in a recent study by Myers et al (19), but this correction has not been made in the methotrexate studies. This type of correction would improve the quantitative reliability of methotrexate studies, particularly in the recovery phase when dUMP pool expansion is likely to be the greatest. Other factors could potentially affect the accuracy of deoxyuridine incorporation including: (a) changes in thymidylate synthetase activity, (b) change in the dTMP pool due to increased or decreased utilization of thymidine via the salvage pathway, or (c) changes in substrate other than thymidine-5'-triphosphate. These factors have not been taken into account in the foregoing studies, but are probably of limited importance in view of the general agreement of most data as to the "threshold" for methotrexate effect.

The Relationship of Drug Concentration and Duration of Exposure to Cytotoxicity

The concept of a "threshold" level of free methotrexate for inhibiting DNA synthesis allowed the first meaningful interpretation of methotrexate pharmacokinetic data. Thus, it was possible for the first time to predict that given levels of drug would be inhibitory to bone marrow or other tissue. Second, it was possible to define resistance to methotrexate in terms of an elevated threshold of sensitivity to the drug, as was observed for human ovarian cancer by Young et al (20). However, the usefulness of the threshold was limited because it provided no information regarding the relative importance of two factors, drug concentration (above the threshold level) and duration of exposure, in determining cytotoxicity. Recent studies in our laboratory in collaboration with Zaharko and Bull (5) have yielded insight into the determinants of cytotoxicity for mouse bone marrow.

In these studies, male C57BL mice received a constant infusion of methotrexate and achieved constant blood levels in a range from 1 x 10^{-8} to 1 x 10^{-5} M. At regular time intervals from 12 to 72 hours after beginning the infusion, groups of animals were killed and femoral bone marrow was removed for cell counting and in vitro cloning in a semi-solid methyl cellulose medium. The number of nucleated cells per femur fell to a nadir 30% of control for all drug concentrations > 10^{-8} M, but the nadir was reached most rapidly at highest concentrations of drug (ie, within 12 hours at 10^{-5} M, 24 hours at 2 x 10^{-6} M, 48 hours at 2 x 10^{-7} M [fig 3], and approximately 72 hours at 5 x 10^{-8} M). The number of myeloid colony-forming units (CFU-C) decreased in parallel to the fall in total nucleated cells during the first 24 hours of infusion, but an increase in the percentage of CFU-Cs per 7.5 x 10^{5} nucleated cells plated was seen during the second 24 hours of infusion at all plasma concentrations, and an absolute increase in the number of CFU-Cs per femur was seen at levels between 2 x 10^{-6} and 10^{-5} M. Although the increased fraction of CFU-Cs at 10^{-7} and 5 x 10^{-8} M could be attributed either to recruitment or to selective depletion of non-colony-forming cells, the absolute increase in CFU-Cs at higher plasma levels constitutes strong evidence for recruitment of precursor cells to myeloid colony-forming potential in response to the cytotoxic agent (21). This recruitment of CFU-Cs probably contributes to the great burst in DNA synthetic activity, far exceeding control rates, which follows recovery from single doses of drug (17).

There is evidence that recruitment of CFU-Cs also occurs after single doses of methotrexate as indicated by the work of Vogler et al (22). In those experiments recruitment started 24 hours after drug administration at a time when little free drug remained, while recruitment during constant high-dose infusion also started between 24 and 48 hrs. Despite continued infusion of drug, Vogler further showed that a knowledge of the pattern of recruit-
ment might be of critical importance in the timing of a repeat dose of chemotherapy.

Previous studies in mice (23) using the spleen-colony assay, in which multiple doses of methotrexate were given daily for 72 hours, also demonstrated a self-limiting toxicity to the hematopoietic stem cells (CFU-S), as observed in the current experiments with CFU-Cs.

To summarize, these experiments with mouse bone marrow clearly demonstrate that the rate of cell-kill is greatest for highest drug concentrations, and that cell depletion is a relatively constant function of time until the nadir of cell-kill is reached. This conclusion correlates well with the empirical observations that small doses of drug, given over long periods of time, are equally as toxic as high single doses of drug (24). The recruitment phenomenon probably occurs with other cytotoxic agents and tends to mitigate the effects of single exposures to myelotoxic agents.

These data provide an opportunity to evaluate the concept that the biologic effects of given schedules of drug administration are a function of concentration \( \times \) time (C \( \times \) t) (25). In this study, as shown in table 1, the C \( \times \) t values for four schedules, all of which produce 70% depletion of nucleated cells from marrow, are markedly different, indicating that biologic effect is not a simple function of C \( \times \) t. Thus, C \( \times \) t value does not bear a constant relationship to the biologic effect of different schedules of methotrexate administration.

**Effect of the Physiologic Environment on Methotrexate Activity**

It would be appropriate at this point to mention several additional aspects of the cellular environment which affect the degree of biologic activity of methotrexate. These factors include the folate cofactor level of the cell, which is probably of importance,
Table 2.—Methotrexate inhibition of CFU-C proliferation and the rescue by nucleosides

<table>
<thead>
<tr>
<th>Additions to bone marrow culture</th>
<th>Colonies (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Methotrexate, 10^{-4}</td>
<td>50</td>
</tr>
<tr>
<td>Methotrexate, 10^{-7}</td>
<td>0</td>
</tr>
<tr>
<td>Methotrexate, 10^{-7} + thymidine, 10^{-4}</td>
<td>0</td>
</tr>
<tr>
<td>Methotrexate, 10^{-7} + thymidine, 10^{-4} + hypoxanthine, 10^{-4}</td>
<td>20</td>
</tr>
<tr>
<td>Methotrexate, 10^{-7} + thymidine, 10^{-4} + hypoxanthine, 10^{-3}</td>
<td>100</td>
</tr>
<tr>
<td>Thymidine, 10^{-4}</td>
<td>20</td>
</tr>
<tr>
<td>Thymidine, 10^{-3}</td>
<td>0</td>
</tr>
</tbody>
</table>

although the level of endogenous folates prior to treatment has not been shown to influence methotrexate sensitivity. Nixon et al (11) have demonstrated a build-up of dihydrofolate in L1210 cells after exposure to methotrexate, and this increase in competitive substrate seems likely to play a role in reversing the inhibition of dihydrofolate reductase in a manner analogous to the reversal ofFdUMP inhibition of thymidylate synthetase by the physiologic substrate dUMP. A second type of reversal has been shown to occur with uracil and pyrimidine nucleosides, the end products of folate metabolism. A combination of thymidine and either hypoxanthine or adenosine has been required in most in vitro systems for full reversal, although purine deprivation appears to play a more important role in cytotoxicity in L5178Y cells (26) than in other cells (27, 28).

Tattersall et al (29) have attempted to improve the therapeutic index of methotrexate with multiple pulse doses of thymidine given every 8 hours for 3 of 4 days after the antifolate. The improvement in therapeutic results seen with combined methotrexate and thymidine was attributed to preferential rescue of normal tissues by thymidine, with continued cytotoxicity for tumor cells due to lack of purines. This interpretation is difficult to reconcile with experiments in tissue culture in which it was clear that both a purine nucleoside and thymidine were required for rescue of mouse bone marrow (table 2); thymidine alone had no rescue effect on the dose/cell-kilogram of methotrexate. The combination of 10^{-4} M thymidine and 10^{-3} M purine nucleoside noncompetitively prevented methotrexate toxicity to bone marrow in culture, but higher concentrations of thymidine alone, or in combination with purines, resulted in toxicity to the CFU in vitro. It is possible that the levels of purine nucleosides circulating in the mouse might be sufficient to allow rescue if supplemented with exogenous thymidine, as in the Tattersall experiment. Little is known, at present, concerning the concentration of circulating nucleosides in man or experimental animals, or the changes in these levels that take place during cytotoxic chemotherapy. In view of the experiments discussed above, one must admit that the relationship between drug level and biologic effect could be altered by changes in nucleoside levels, and this may prove a fruitful area for further investigation.

To summarize, this paper has dealt with our present understanding of the determinants of methotrexate cytotoxicity, specifically the role of free-drug concentration, duration of exposure, and the physiologic environment, including folate and nucleoside pools. In order to provide a rational basis for chemotherapy in man, additional information is needed regarding these factors and their relationship to cytotoxicity for normal and malignant tissues in man.

REFERENCES


