Cytokinetic Comparison of Thymidine and Leucovorin Rescue of Marrow in Humans after Exposure to High-Dose Methotrexate

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ABSTRACT

The cytokinetics of marrow recovery were compared in patients receiving a standard exposure to high-dose methotrexate followed by either thymidine rescue, leucovorin rescue at the doses used in most clinical protocols (10 mg/sq m every 6 hr), or leucovorin rescue at a 5-fold higher dose rate (50 mg/sq m every 6 hr). Thymidine rescue initiated a prompt recovery of DNA synthesis, as detected by [3H]deoxycytidine incorporation, and progression of cells through the cell cycle monitored by flow cytometry, even in the presence of methotrexate levels that prevented initiation of rescue by the lower doses of leucovorin. Dose dependency for leucovorin in vivo in humans was suggested by the observation that the higher leucovorin dose rate was successful in initiating rescue within the first 24 hr, whereas the lower dose was not. Recovery of DNA synthesis is more rapid and/or complete with thymidine rescue than with either dose of leucovorin. Thymidine rescue was accomplished without requirement for purines over and above those present in plasma. These results suggest that the kinetics of marrow recovery is quite different for thymidine and leucovorin rescue.

INTRODUCTION

MTX is thought to exert cytotoxicity by causing depletion of intracellular reduced folates required as cofactors for the de novo synthesis of thymidylate and purines (24, 45). The biochemical action of MTX can be reversed either by restoring reduced cofactor pools with leucovorin (7) or by providing cells with the end products of the inhibited pathways, thymidine and purines (1, 10, 11, 31). Leucovorin is transported into cells by the same facilitated transport system that carries MTX (9), and in tumor models the ability of leucovorin to rescue MTX is dose dependent and competitive with MTX (1, 31, 37, 38). However, dose dependency for leucovorin rescue has not been clearly demonstrated in humans. Studies of mouse marrow in vitro indicate that reversal of MTX cytotoxicity by thymidine is dose dependent but not competitive with MTX (31). In most but not all in vitro and murine systems, a source of purines as well as thymidine is required for effective rescue (1, 10, 12, 31, 34-36, 39, 41, 43, 45).

Thymidine is of interest as a rescue agent because of the possibility that it may provide a more selective rescue than is achievable with leucovorin. In vivo experiments with the murine leukemia L1210 indicate that the salvage pathway metabolite requirements of the malignant cells are sufficiently different from those of normal mouse tissues that concurrent administration of MTX and thymidine permits expression of the antitumor action of MTX while simultaneously protecting the host (4, 12, 35, 36, 41). In a previous Phase 1 trial, we examined the possibility of using thymidine as a rescue agent in humans (16). Thymidine effectively rescued MTX in doses of up to 8.5 g/sq m, representing a greater than 70-fold increase in the maximum tolerated dose. In the study reported here, we compared the rate of rescue of marrow mononuclear cells in patients receiving either thymidine or leucovorin rescue following a standard exposure to MTX. Independent biochemical and cytokinetic techniques were used to measure the recovery of DNA synthesis and progression of cells through the cell cycle. The results demonstrate that, in humans, thymidine can initiate rescue of marrow cells in the presence of plasma concentrations of MTX that prevent initiation of rescue by the standard doses of leucovorin used in many clinical programs. Rescue with thymidine appears to be more rapid and/or more complete than rescue with leucovorin.

MATERIALS AND METHODS

Patients and Treatment Program. Fifteen courses of high-dose MTX therapy were given to 12 patients with histologically proven advanced cancer, who gave informed consent to enter this study. All patients received an i.v. push of 0.4 g/sq m of MTX to establish an MTX level in the range of 10⁻⁴ M, and then this level was maintained by the constant infusion of 3.375 g/sq m over the next 24 hr. At 24 hr, the MTX infusion was stopped and rescue was begun. Thymidine was used as the rescue agent on 4 courses and was given as a constant infusion of 8 g/sq m/day until the serum MTX level was less than 5 × 10⁻⁸ M. On 7 courses, leucovorin was used at a dose of 10 mg/sq m given every 6 hr for 4 doses and then 50 mg/sq m every 6 hr for an additional 8 doses. The dose rate of 10 mg/sq m every 6 hr is the standard dose of leucovorin used by most high-dose MTX treatment programs (19, 29). On 4 other courses, the leucovorin rescue program was used at a 5-fold greater dose of 50 mg/sq m every 6 hr for 12 doses. With both dose schedules, the first dose was given i.v. and subsequent doses were given p.o. All patients had a creatinine clearance of greater than 60 ml/min. Serial posterior iliac crest bone marrow aspirations were obtained with a Jamshidi needle immediately prior to therapy and at 24 and 48 hr after the start of MTX exposure. All patients received
NaHCO₃ (3.0 g p.o.) every 3 hr to alkalize the urine. Bicarbonate was started 12 hr before and continued for 48 hr after the start of MTX. MTX was not administered unless the urine pH was greater than 7.0. Urine output was maintained at greater than 3 liters/day during the MTX infusion.

**Measurement of Serum MTX and Thymidine Levels.**

MTX and thymidine levels were measured by radioimmunoassay as previously described (18, 33).

**Bone Marrow Studies.** Two ml of bone marrow were immediately anticoagulated with 30 mg of EDTA, the sample was divided in half, and without further dilution it was incubated with 1.0 μCi [3H]dUrd or [3H]Cyd for 1 hr (New England Nuclear, Boston, Mass.; deoxycytidine specific activity, 27.8 Ci/mmol; deoxycytidine specific activity, 60 Ci/mmol). Samples were then diluted with cold Hanks' balanced salt solution, placed on a Ficoll-Hypaque gradient, and centrifuged at 750 x g for 20 min. Marrow mononuclear cells recovered from the Ficoll-Hypaque interface were washed twice in Hanks' balanced salt solution and counted with a Model ZF Coulter counter. An aliquot of each sample was precipitated with 10% trichloroacetic acid, collected on Whatman GF/C filters, and prepared for scintillation counting as described elsewhere (20).

In preparation for flow cytometric analysis, mononuclear cells recovered from the Ficoll-Hypaque gradient were centrifuged and cell pellets resuspended in 5 ml of propidium iodide/hypotonic citrate solution (propidium iodide, Calbiochem, San Diego, Calif.; 0.05 mg/ml in sodium citrate) (20). After 15 to 30 min of staining, samples were analyzed for DNA content per cell on a Model 4801 Cytofluorograph (Ortho Instruments, Westwood, Mass.), fitted with an external stabilized photomultiplier supply and improved sheath water flow. DNA histograms were generated from analysis of 40,000 cells/sample in a pulse height analyzer. Details of this instrument have been published earlier (21, 22).

**RESULTS**

Chart 1 shows that the serum MTX levels attained during the MTX infusions were similar during courses of treatment on which either thymidine, low-dose leucovorin, or high-dose leucovorin rescue was used. The levels ranged from a low of 2 x 10⁻⁴ to a high of 1 x 10⁻³ M with a mean of 1 x 10⁻⁴ M. The thymidine concentration during rescue averaged 1 x 10⁻⁶ M. No patient developed any form of MTX-induced toxicity on any of the courses administered in this study.

The incorporation of [3H]dUrd into the acid-insoluble fraction from 10⁶ marrow mononuclear cells was used to estimate the degree of inhibition of DHFR produced by MTX. Although both inhibition of DHFR and expansion of intracellular thymidylate pools by the exogenously administered thymidine can suppress [3H]dUrd incorporation, the latter effect is minimal at the thymidine concentrations achieved in this study during rescue (40). To measure the rate of DNA synthesis, the incorporation rate of [5-3H]Cyd was chosen since cytidine enters DNA without going through a folate-dependent step. To exclude the possibility that some of the 3H label was being incorporated into RNA, the number of counts in the acid-insoluble fractions of marrow cells from 4 patients were compared before and after the RNA was removed with a Schmidt-Thannheuser extraction (26). There was no loss of counts in the acid-insoluble fraction following removal of RNA, either before or after exposure to high-dose MTX in vivo.

Chart 2 shows that at 24 hr, i.e., at the end of the MTX infusion and just before the administration of rescue, the rate of [3H]dUrd incorporation into bone marrow cells was severely depressed on all courses, compared to the rate measured just prior to the start of the MTX infusion. When thymidine was used as the rescue agent, there was no increase in the [3H]dUrd incorporation during the first 24 hr of rescue, consistent with the continued presence in the plasma of MTX levels high enough to inhibit dihydrofolate reductase, although possibly reflecting some expansion of intracellular thymidylate pools. When leucovorin in standard doses was used as the rescue agent, there was little evidence of recovery of [3H]dUrd incorporation on most courses. However, recovery was evident on some courses when high levels of leucovorin were used.

As shown in Chart 3, bone marrow DNA synthesis as reflected by [3H]Cyd incorporation was also depressed at the end of the 24-hr MTX infusion, but only to a mean of...
Thymidine and Leucovorin Rescue

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24.6% of the pretherapy value. In contrast to its lack of effect on \(^3\)H)dUmd incorporation, thymidine rescue resulted in a marked increase in \(^3\)H]Cyd incorporation during the first 24 hr of rescue, indicating resumption of DNA synthesis despite continued high-blood MTX levels. Standard doses of leucovorin had no effect on \(^3\)H]Cyd incorporation during the first 24 hr, whereas a 5-fold higher dose rate of leucovorin was effective in initiating an increase in DNA synthesis. These results demonstrate dose dependence for the initiation of rescue by leucovorin in humans. It is noteworthy that at the dose administered in these studies, thymidine appeared to produce a greater recovery of DNA synthesis after 24 hr of rescue, as measured by \(^3\)H]Cyd incorporation, than was accomplished with even the high doses of leucovorin.

In order to confirm the results of these isotope incorporation studies, the independent technique of flow cytometry was used to analyze the distribution of cells in the cell cycle. Chart 4 demonstrates the serial changes observed in a representative patient who received thymidine rescue on one course and standard doses of leucovorin (10 mg/sq m every 6 hr) on another course of MTX therapy.

The DNA histograms of nucleated marrow cells were normal prior to each course of MTX therapy in all patients, similar to the histogram shown in Chart 4A. There was a prominent G, peak centered about Channel 150, G, - M peak at Channel 300, and a small population of S cells between the 2 peaks. After 24 hr of MTX exposure, the DNA histograms from all patients demonstrated accumulation of cells at the G, - early S interface (arrow) with a corresponding decrease of cells in the late-S and G, - M phases as shown in Chart 4B. On the courses where either thymidine or the high doses of leucovorin were used as the rescue agent, the cytometric analysis showed that cells were no longer accumulated at G, - early S interface, and that marrow cells with the DNA content of late S and G, - M had reappeared in the population by 48 hr as seen in Chart 4C. However, on the courses where standard doses of leucovorin were used, the marrow cells remained blocked at the G, - early S interface (with a corresponding reduction in G, - M) as shown in Chart 4D. Although there was some variation in the degree of G, - early S phase accumulation at 24 hr from patient to patient, the pattern of response to the rescue agent was clear in each case. These results confirm those of the DNA precursor incorporation studies, indicating that both thymidine and high-dose leucovorin were effective in initiating rescue as evidenced by the progression of cells through S phase, whereas standard low doses of leucovorin were ineffective, despite a full 24 hr of rescue with this particular program of MTX administration.

DISCUSSION

A cytokinetic technique, flow cytometry, and a biochemical technique, incorporation of labeled DNA precursors, were used in this study to compare the ability of thymidine
and leucovorin to initiate rescue in human marrow cells in vivo following exposure to high-dose MTX.

There are a number of difficulties with the use of [3H]dUrd incorporation to measure MTX-induced inhibition of dihydrofolate reductase and [3H]Cyd incorporation to measure blockade of DNA synthesis. MTX induces major changes in intracellular nucleotide pools (42) that are quite variable from tissue to tissue (43) and may influence the specific activity of labeled precursor reaching the nucleotide pool. This may be particularly true for the combination of MTX with thymidine (42), both of which can independently alter deoxycytidine pools. Likewise, expansion of intracellular TTP pools by exogenous administration of thymidine may stimulate phosphorylation of [3H]Cyd (32). In addition, deoxycytidine can be deaminated to deoxyuridine and then converted to thymidine by thymidine synthetase and hence incorporated into DNA. This latter problem was avoided in these studies by using [5-3H]Cyd, since the 5-3H label is lost in the thymidine synthetase reaction (23). Our observation that incorporation of [3H]Cyd is limited primarily to DNA is in agreement with observations of others on Novikoff hepatoma cells (32) and human marrow cells (4).

Because of these uncertainties, it is important that a completely separate technique be used to corroborate the results. In these studies, we used flow cytometry for this purpose. Flow cytometry measures the amount of DNA in individual nuclei based on the amount of fluorescence emitted by propidium iodide bound quantitatively to nucleic acid (20). The relative proportions of cells in each phase of the cell cycle can be estimated from DNA histograms, and the results agree closely with the labeling index (21). The changes in the flow cytometric pattern associated with exposure to and recovery from MTX have been well described (22) and are the same as the patterns observed in this study. We found that the flow cytometric analysis of marrow cells during rescue provided good confirmation of the results obtained with incorporation of labeled nucleosides.

The major conclusion from these results is that thymidine can initiate recovery of DNA synthesis in human marrow cells in the presence of concentrations of MTX that prevent the initiation of rescue by the standard doses of leucovorin used in a majority of the clinical high-dose MTX treatment programs. Rescue with thymidine is noncompetitive with MTX in tissue culture systems (1, 31) consistent with the separate membrane transport systems for MTX and thymidine. The ability of thymidine to initiate DNA synthesis in the presence of 1 x 10^{-4} M MTX suggests that the same may be true in humans in vivo.

In contrast to thymidine, tissue culture studies demonstrate that leucovorin must compete with MTX for transport into cells (8, 9) and that dose dependency for the ability of leucovorin to rescue as a function of MTX serum level has been shown in murine systems (31, 37, 38). Our results suggest dose dependence for leucovorin rescue in vivo in humans, but further studies would be required at multiple MTX concentrations in order to demonstrate true competition. Administration of standard doses of leucovorin, 10 mg/sq m every 6 hr, failed to produce any evidence that DNA synthesis was resuming during the first 24 hr after the start of rescue. On the other hand, high-dose leucovorin, 50 mg/sq m every 6 hr, was successful in initiating DNA synthesis within the first 24 hr as evidenced both by [3H]Cyd incorporation and flow cytometric analysis. It is important to note that standard-dose leucovorin might have resulted in adequate clinical rescue since resumption of DNA synthesis beyond the first 24 hr after the start of rescue may still possibly prevent MTX toxicity (2). However, because previous clinical studies have suggested that unrescued exposure to MTX for longer than 36 to 42 hr is frequently associated with toxicity (6), in the investigation reported here the dose rate of leucovorin was increased from 10 to 50 mg/sq m after the first 24 hr of rescue on all 7 courses where the lower dose rate was used initially.

When examined in tissue culture, most cells require a source of purine as well as thymidine to protect them against the cytotoxic effects of MTX (1, 4, 17, 24, 31, 43). In in vivo trials with mice, some (12, 39), but not all (35, 41) investigators found that infusion of hypoxanthine or inosine in addition to thymidine was required to fully protect gut. The data presented here and the results of our previous studies (16, 33) indicate that in humans thymidine alone is adequate to rescue normal tissues, and that there is no need for an additional source of purines over and above that available in human plasma. This species difference may be due to higher circulating levels of purines in humans or relatively less inhibition of de novo purine as opposed to pyrimidine synthesis by MTX (14, 27, 28, 46).

The actual levels of thymidine and preformed purines are probably important in determining the selectivity of MTX. Exposure of bone marrow cells to a mean level of 1 x 10^{-4} M MTX in vivo, although it caused a major shift in the distribution of cells into G_0-early S, reduced the quantitative incorporation of [3H]Cyd to a mean of only 24.6% of the pretherapy value. One explanation for this may be the presence of enough salvage pathway metabolites to partially block MTX cytotoxicity. Pinedo et al. (31) and Mitchell et al. (25) observed that MTX was unable to completely inhibit murine marrow stem cell proliferation in the presence of undialyzed fetal calf serum, and they attributed this to the presence of high levels of salvage pathway metabolites. If there are sufficient salvage metabolites present in normal marrow to block MTX cytotoxicity partially, then higher than normal local concentrations of these metabolites in tumors may completely block the action of MTX (5, 31, 44). This may be an important mechanism of resistance to MTX in tumor nodules in vivo where there is continuous cell death and catabolism of nucleic acids.

The recovery of DNA synthesis measured by [3H]Cyd incorporation was higher after 24 hr of rescue in patients receiving thymidine than in patients receiving either dose rate of leucovorin. It is probable that this reflects more rapid recovery of DNA synthesis with thymidine rescue, but it may also relate to differences in recruitment of previously uncommitted precursor cells into cycle by thymidine and leucovorin (30). Interpreting higher rates of DNA synthesis as indicative of more rapid or complete recovery of the ability to withstand subsequent exposure to MTX requires caution. It has yet to be demonstrated that MTX with thymidine rescue can be given on as frequent a dose schedule as MTX with leucovorin rescue.

The primary reason for interest in thymidine is the hope
that it will prove to be a more selective rescue agent than leucovorin. Whether or not this turns out to be true, the results of these studies indicate other possible uses for thymidine rescue: (a) the ability of thymidine to initiate rescue in the presence of very high levels of MTX suggests that thymidine may be more reliable and effective than leucovorin in salvaging patients who develop acute renal failure and maintain very high MTX plasma concentrations for prolonged periods; (b) if marrow recovery is more rapid with thymidine, it may permit more frequent administration of MTX and a higher dose rate; (c) thymidine rescue may be useful in exploring any therapeutic advantage to be gained by long duration infusions of MTX with abrupt termination of drug effect; (d) where desired, such abrupt reversal of the action of MTX may produce much better marrow synchronization than is achievable with other agents in vivo.

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