Potentiation of the Antitumor Activity of Methotrexate by Concurrent Infusion of Thymidine

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ABSTRACT

The effects of normal metabolites on the toxicity and antitumor activity of methotrexate against leukemia L1210 in DBA/2J mice were evaluated by a system that allows for long-term continuous i.v. infusion of unrestrained mice. In normal female DBA/2J mice, the infusion of methotrexate alone for 48 hr produced a 50% lethal dose of 6 mg/kg/day. Coadministration of thymidine (5 g/kg/day) and methotrexate, followed by an additional 48 hr of thymidine alone, dramatically reduced toxicity, resulting in a 50% lethal dose of about 45 mg/kg/day. A higher concentration of thymidine (18 g/kg/day), which was only marginally toxic alone, was even more effective and reduced the toxicity of methotrexate more than 35-fold.

Against leukemia L1210 in female DBA/2J mice, a 48-hr infusion of methotrexate produced a 33% increase in life span at the optimum dose of 1 mg/kg/day. The addition of thymidine (5 g/kg/day) to the methotrexate for 48 hr potentiated antitumor activity and resulted in a maximum 68% increase in life span with methotrexate at 4 mg/kg/day. At higher concentrations of methotrexate, two additional days of thymidine infusion were required for prevention of toxicity while maintaining antitumor activity. Maximum therapeutic selectivity and a 125% increase in life span were obtained with methotrexate at 16 mg/kg/day, infused for 48 hr concurrently with thymidine at 5 g/kg/day for 96 hr. A higher concentration of thymidine (15 g/kg/day), although affording a greater than 35-fold reduction in toxicity, also prevented antitumor activity.

The infusion of inosine alone or in combination with thymidine blocked both the toxicity and antitumor activity of methotrexate. These results indicate that the increase in the therapeutic selectivity achieved with the simultaneous infusion of methotrexate and thymidine may result from a complex modulation of cellular metabolism rather than simple end product reversal by the provision of thymidylate.

INTRODUCTION

Hakala (9) and Hakala and Taylor (10) using Sarcoma 180 in cell culture were the first to demonstrate that the lethal effects of MTX could be prevented by the simultaneous provision of the end products of folate metabolism: dThd, a purine such as hypoxanthine, and glycine. In these cells the addition of purine alone to the culture medium afforded a 2-fold decrease in the toxicity of MTX; whereas the addition of dThd alone was ineffective. In a subline of Sarcoma 180 selected for resistance to MTX, the addition of hypoxanthine to the unsupplemented medium had no MTX-sparing effect. However, the addition of dThd alone allowed a 4-fold decrease in toxicity (11). More recently, a similar dThd-sparing effect was observed by Borsa and Whitmore (3) using a subline of L-cells (L60T). In contrast, in L5178Y cells hypoxanthine but not dThd yielded partial protection from MTX toxicity (13). Thus, various investigators have concluded that in some cell inhibition of purine nucleotide synthesis is most critical, whereas other investigators have attributed MTX growth inhibition primarily to a thymidylate deprivation.

Grindey and Moran (7) demonstrated in vivo that the antitumor activity but not the toxicity of MTX was reversed by coadministration of allopurinol. Previous work by Pomales et al. (29) had shown that the incorporation of purines into cellular nucleic acids in the mouse could be dramatically increased by this agent via inhibition of xanthine oxidase, a purine-degradative enzyme. Therefore, the antagonism by allopurinol of the antitumor effect of MTX may have been due to increased utilization of salvage purines such as hypoxanthine by the tumor. Preliminary studies involving injection of dThd phosphorylase into mice revealed that depletion of salvageable dThd had no effect on the therapeutic efficacy of MTX against leukemia L1210 (8). The enzyme treatment did, however, increase the toxicity of MTX to the host. Further in vivo experiments by Tatterstall et al. (35) evaluated the effects of dThd on the antitumor activity of MTX. The injected dThd allowed some protection from MTX toxicity in normal mice and afforded a 44% increase in life span to L1210-bearing animals. However, the MTX alone on the same schedule was therapeutically ineffective (35). Thus, the antileukemic activity of MTX in vivo may be more related to a purineless death, whereas toxicity to normal target tissues may be attributed to a thymidylate limitation. Based on these in vivo and cell culture results, this study sought to investigate the effects of salvage metabolites administered by continuous i.v. infusion on the toxicity and antitumor activity of MTX in the mouse. The administration of normal metabolites by infusion allows the establishment of constant, steady-state plasma levels similar to cell culture conditions.

MATERIALS AND METHODS

Female DBA/2J and C57BL/6 mice (20 to 22 g) were purchased from The Jackson Laboratory, Bar Harbor, Maine. The leukemia L1210 was used as described previously (23, 32). In all experiments 10⁶ L1210 cells were inoculated i.p. into recipient mice, and drug infusions were
begun 24 hr later. The mean survival was calculated from the
day of tumor inoculation, which was considered to be
Day 0.

The drugs were obtained from Sigma Chemical Co., St.
Louis, Mo., or Lederle Laboratories, Pearl River, N. Y.
Drugs were administered via continuous i.v. infusion in
unrestrained mice at a rate of 0.37 ml/hr. The technique
was modified from that of Paul and Dave (27) to allow
simultaneous infusion of 40 mice for up to 6 days with 5
Harvard Model 940 infusion pumps (Harvard Apparatus Co.,
Inc., Millis, Mass.) equipped with adapters for holding 8
glass syringes (5 ml). Disposable plastic syringes (12 ml;
Sherwood, Deland, Fla.) fit these adapters and do not
freeze during the long-term infusions. The shields and
splints were constructed as described by Paul and Dave (27).

After the mouse is restrained, a lateral tail vein is punctured
about 2.5 cm from the base of the tail with an 18-gauge
needle and then cannulated with Intramedic P. E. 10 poly-
ethylene tubing. The tubing is inserted into the vein to about
1 cm from the base of the tail. After spraying the tail lightly
with surgical dressing, it is wrapped with 0.5-inch gauze and
sprayed again with dressing to form a protective cocoon.
The tail is taped to the splint, the shield is positioned,
and the mouse is put into a 6-×10-inch cage. The splint
is attached by wire to an overhead support to keep the
tail roughly perpendicular to the cage floor. The other end
of the cannula is attached to a syringe with either a 27.5-
or 30-gauge needle. Drugs were dissolved in either 0.9%
NaCl solution or reduced salt to maintain isotonicity and
adjusted to pH 7 to 7.4. Prior to administration all solutions
were sterilized via passage through 0.22-μm Millipore
filters.

The procedure for identification of plasma salvage metab-
olites by HPLC (Model 830: DuPont Instruments, Des
Plaines, Ill.) is an early modification of that described by
Rustum (31). Separation is achieved on a Zorbax ODS
column eluted with 2.5 mM KH₂PO₄, pH 6.9. After centrifu-
gation to remove RBC, the plasma was extracted with
perchloric acid (final concentration, 4.4%), and the extract
was neutralized with 1 N KOH and analyzed with HPLC by
injection of 10 μl of the extracted sample.

RESULTS

Nucleoside Reversal of MTX Toxicity. A comparison of
MTX toxicity in normal female DBA/2J and C57BL/6 mice
indicated that the 2 strains exhibited a slight difference in
sensitivity to 48 hr of continuous infusion of MTX alone
(Chart 1). Whereas the 50% lethal dose of 6 and 11 mg/kg/
day for the 2 strains was not statistically different, the
difference in the 100% lethal dose between the 2 strains
was reproducible in subsequent experiments. The lack of
statistical significance for the 50% lethal dose values is
related to the more shallow dose response curve obtained
with the C57BL/6 mice. Simultaneous infusion of dThd (5
g/kg/day) and MTX for 48 hr, followed by an additional 48
hr of dThd alone, produced about 8- and 5-fold shifts in
the MTX survival curves in the DBA/2J and C57BL/6 mice,
respectively. This regimen provided significant protection
(p < 0.05) from MTX toxicity in both strains of mice. dThd
was infused for an additional 2 days after termination of the
MTX to provide thymidylate during the resynthesis of dihy-
drofolate reductase in tissues (12). The infusion of a lower
concentration of dThd (1 g/kg/day) was relatively ineffect-
ive in reducing MTX toxicity (p > 0.05). High doses of
concurrent dThd (18 g/kg/day) for a total of 96 hr afforded
almost complete protection against mortality in both strains
with MTX up to 140 mg/kg/day, a 35-fold decrease in
toxicity. This regimen was significantly (p < 0.05) more
effective than the lower concentration of dThd in reducing
MTX toxicity. Infusion of this concentration of dThd alone
for 96 hr produced some toxicity in normal mice as evid-
ced by a 3.2-g weight loss at the end of infusion and a
20% mortality. Slightly lower doses of dThd (15 g/kg/day)
alone yielded no gross toxicity.

Further studies were carried out on the modulation of
MTX toxicity by salvageable nucleosides in normal female
DBA/2J mice. As shown in Chart 1 and listed in Table 1, a
48-hr infusion of MTX at 32 mg/kg/day resulted in 0%
survival. Coadministration of dThd for 96 hr offered partial
although significant (p < 0.05) protection with 80% of the
mice surviving and an average weight loss of 4.2 g/mouse.
Infusion of both end products of folate metabolism, dThd
and a source of purine, inosine, at optimal doses signific-
antly (p < 0.05) prevented lethal toxicity and minimized
weight loss. Lowering the concentration of dThd produced
increased weight loss (Table 1). The infusion of inosine (4
g/kg/day) alone for 96 hr concurrently with MTX was also
capable of significantly (p < 0.05) reducing mortality;
however, a large weight loss was evident and the animal’s
appearance indicated that full reversal of MTX toxicity had
did not occur. By using the same scheduling as in Table 1,
the toxicity of MTX at 64 mg/kg/day could be significantly
(p < 0.05) reversed with inosine alone or dThd plus inosine;
however, dThd (5 g/kg/day) alone could not prevent mort-
ality.

Effects of Normal Metabolites on the Antileukemic Ac-
tivity of MTX. Chart 2 demonstrates the effects of concur-
rent infusion of various doses of dThd on the antitumor
activity of a single concentration of MTX against leukemia
L1210 in female DBA/2J mice. MTX (4 mg/kg/day) infused
alone for 48 hr, although a nonlethal dose in normal mice,
is stressful to the tumor-bearing animals, which die ahead

![](chart1.png)

Chart 1. Effects of dThd (TdR) on the toxicity of MTX in normal DBA/2J
(closed symbols) and C57BL/6 (open symbols) female mice. MTX was
infused alone for a total of 48 hr. The combination of dThd (5 or 18 g/kg/
day) and MTX was infused for 48 hr, and then the dThd was continued alone
for an additional 2 days. Each group contained 5 mice. Each of the regimens
provided a significant (p < 0.05) reduction in MTX toxicity as determined by
the procedure of Mantel and Haenszel (22).

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of controls (Chart 2). Doses of dThd below 1 mg/kg/day were not sufficient to reverse MTX toxicity substantially. At this concentration of MTX, a significant (p < 0.05) therapeutic effect was achieved with dThd at 2.5 g/kg/day, allowing an average survival of 12.8 ± 1.8 days (S.D.). As the dose of concurrent dThd was increased to 18 g/kg/day, significant (p > 0.05) antitumor activity was abolished. Infusion of similar high levels of dThd alone had no significant (p > 0.05) antitumor activity. Although maximum survival was obtained with dThd at 2.5 g/kg/day, the higher dose of dThd (5 g/kg/day) was used in subsequent antitumor experiments, for it provided better toxicity reversal and minimal weight loss.

The effects of concurrent infusions of normal metabolites on the antileukemic activity of MTX are summarized in Table 2 and Chart 3. The infusion of MTX alone for 48 hr is rather ineffective against L1210, yielding only a 33% increase in life span over untreated leukemic mice.

Table 1

<table>
<thead>
<tr>
<th>% of survival</th>
<th>Av. wt loss (g)</th>
</tr>
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<tbody>
<tr>
<td>MTX alone</td>
<td>0</td>
</tr>
<tr>
<td>MTX + dThd (5 g/kg/day)</td>
<td>80</td>
</tr>
<tr>
<td>MTX + inosine + dThd (5 g/kg/day)</td>
<td>100</td>
</tr>
<tr>
<td>MTX + inosine + dThd (1 g/kg/day)</td>
<td>100</td>
</tr>
<tr>
<td>MTX + inosine</td>
<td>100</td>
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a Mean ± S.D. of weight loss for each group at the end of drug treatment.

The inosine (4 g/kg/day) produced no gross toxicity and minimal weight loss when infusion alone was 96 hr.

Chart 2. Effects of varying concentrations of dThd (TdR) on the antitumor activity of 1 dose of MTX against L1210 in DBA/2J female mice. The drugs were administered on Day 1, 24 hr after tumor inoculation. The average survival time of the mice treated with MTX (4 mg/kg/day) alone is 6.8 ± 0.5. MTX and dThd were infused for 48 hr, and then the dThd was continued alone for an additional 2 days. Each group contained 5 mice. Controls, average survival time of untreated leukemic mice of 8.4 ± 0.5. The average survival (12.6 ± 1.8) of the optimum combination was significantly (p < 0.05) better than controls or MTX alone as determined by the analysis of Breslow (4).

Chart 3. Summary of the antitumor effects of various MTX-dThd regimens against leukemia L1210 in DBA/2J mice. All drugs were administered by infusion starting 24 hr after L1210 inoculation. MTX alone was infused for 48 hr (□). Either the combination of MTX plus dThd (5 g/kg/day) was infused for 48 hr (■), or the dThd was continued alone for an additional 2 days (△). The combination of MTX plus dThd (15 g/kg/day) was infused for 48 hr with the dThd continued for 2 additional days (○). Each group contained 5 mice, except the MTX plus dThd (5 g/kg/day) groups that are the summary of 2 replicate experiments of 5 mice each. Controls, average survival time of untreated leukemic mice.

increase in life span over controls (Chart 3). An optimum schedule of MTX (i.p., 3 doses on alternate days) previously yielded a 73% increase in life span over controls with this tumor in DBA/2J mice (6). Coadministration of dThd and MTX for 48 hr potentiated MTX activity, allowing a 14-day average survival time with MTX at 4 mg/kg/day. At higher concentrations of MTX, the 2 extra days of dThd infusion are necessary for prevention of toxicity while maintaining MTX antitumor activity. Maximum therapeutic selectivity and an increase in life span of 125% were obtained with MTX at 16 mg/kg/day infused 48 hr concurrently with dThd (5 g/kg/day) for 96 hr. As the MTX concentration was further increased, the mice died of toxicity. In contrast, concurrent infusion of dThd at 15 g/kg/day prevented toxicity and weight loss at high doses of MTX; however, it reversed tumor kill also and no increase in survival was achieved. As shown in Chart 4, survival curves for the various regimens were constructed at the optimum concentration of MTX for each regimen to facilitate statistical analysis as described by Breslow (4). The infusion of
MTX alone at 1 mg/kg/day for 48 hr produced significant \( (p < 0.05) \) antitumor effects with an average survival of 11.0 ± 1.1 days as compared to 8.7 ± 0.6 for untreated controls. The coinfusion of dThd (5 g/kg/day) with the MTX (4 mg/kg/day) for 48 hr is significantly \( (p < 0.05) \) more effective than MTX alone against L1210, resulting in an average survival time of 14.2 ± 2.8 days. When the infusion of dThd (5 g/kg/day) was continued for 48 hr beyond the MTX (16 mg/kg/day), the antitumor effects were significantly \( (p < 0.05) \) increased beyond the previous combination, with an average survival time of 19.6 ± 2.6 days in this experiment. No significant \( (p > 0.05) \) antitumor effects were observed at this concentration of MTX when the concentration of dThd was increased to 15 g/kg/day on the same schedule.

Table 2 demonstrates the reversal of antileukemic activity by coadministration of inosine with the MTX-dThd combination. The infusion of inosine alone with the antifolate blocked most antitumor activity with MTX at 1 and 2 mg/kg/day, consistent with the previous results obtained with allopurinol (7). At high doses of MTX in the leukemic mice, the inosine alone was not capable of preventing toxic deaths and the mice died ahead of controls (Table 2). This is consistent with the severe weight loss observed in normal mice infused with MTX at 32 mg/kg/day plus purine alone (Table 1).

**Concentration of Salvage Metabolites in Plasma during Infusions.** In normal DBA/2J plasma, the concentration of hypoxanthine as determined by HPLC is approximately 1 to 2 \( \mu M \), whereas that of inosine is 20 \( \mu M \). Although the thymine concentration is below detection \( (<1 \mu M) \), the concentration of dThd is about 0.5 to 1 \( \mu M \), consistent with the previous report by Hughes et al. (14) using a radioimmune assay to measure serum dThd. As shown in Chart 5, the infusion of dThd at 5 g/kg/day dramatically increased the circulating level of thymine as well as dThd and achieved a plasma concentration of dThd of about 100 \( \mu M \). Infusion of this dose of dThd did not substantially alter the concentration of other salvage metabolites in plasma. Similar results were observed in replicate experiments and when this dose of dThd was infused with MTX (32 mg/kg/day). However, the infusion of dThd at 15 g/kg/day increased the concentration of hypoxanthine and other metabolites in plasma in addition to that of dThd and thymine (Chart 5). The approximate plasma concentrations of metabolites in this and replicate experiments were: dThd, 800 \( \mu M \); thymine, 400 \( \mu M \); inosine, 20 \( \mu M \); and hypoxanthine, 13 \( \mu M \). Increased levels of purines were also observed in plasma when this concentration of dThd was infused with MTX (140 mg/kg/day).

**DISCUSSION**

Effects of End Products on MTX Activity. Whereas, in cell culture, extensive data have been generated on the biological effects of salvage metabolites on MTX activity, the in vivo evaluation of these observations has been complicated by the short plasma half-life of the metabolites (5, 30). Their administration by continuous i.v. infusion results in the establishment of constant, steady-state plasma levels similar to cell culture conditions and allows a direct translation of cell culture results to the in vivo situation. As shown in Tables 1 and 2, concurrent infusion of dThd and a source of salvageable purine, inosine, with MTX completely reversed both toxicity and antitumor activity in vivo comparable to what was previously observed in cell culture (3, 8–10, 36). Concurrent infusion of inosine alone also blocked MTX-induced lethality (Table 1). This somewhat surprising observation is probably a consequence of the 0.5 to 1 \( \mu M \) plasma dThd in the mouse (see "Results" and Ref. 14). In the presence of adequate purine, this concentration of dThd is able to partially reverse the growth inhibitory effects of MTX on mammalian cells in culture, with optimum reversal occurring at 6 to 10 \( \mu M \) (7, 28). The substantial weight loss (4.7 g) observed following the infusion of inosine and MTX indicated that only a partial reversal of toxicity was achieved, as might be expected in the presence of this low concentration of dThd. The minimal weight loss observed following the infusion of additional dThd with this combination (Table 1) and the lack of significant antitumor activity in the presence of inosine...
would be consistent with the total loss of MTX antitumor activity (36). The addition of dThd alone partially reversed the simultaneous infusion of high doses of dThd. protection was incomplete, similar to that observed in the cultured cell lines, and survival decreased with increasing dose of MTX. With MTX at 64 mg/kg/day, the lower dose of concurrent dThd no longer afforded any reversal of mortality (Chart 1). This MTX dose dependence for survival is inconsistent with the postulate of end product reversal of its effects produced by infusion of dThd and salvage of naturally occurring purines by the normal tissues. Via this mechanism even the highest doses of MTX should have been nontoxic (3, 8-10, 36).

The infusion of larger doses of dThd (15 g/kg/day) was more effective in reducing the toxicity of high doses of MTX, although antitumor activity was blocked also. As shown in Chart 6, however, the infusion of this high concentration of dThd altered the level of other salvage metabolites such as hypoxanthine in addition to increasing the plasma concentration of dThd and thymine. The mechanism of this induced increase in circulating purines is not understood. The concentrations of dThd and hypoxanthine observed under these conditions should be sufficient to achieve a total end product reversal of MTX toxicity. This would be consistent with the total loss of MTX antitumor activity and toxicity observed in Charts 1 to 3 following simultaneous infusion of high doses of dThd.

**Metabolic Modulation of MTX Activity.** Another mechanism that could account for the ability of dThd to partially reverse MTX toxicity with retention of antitumor activity involves the indirect inhibition of thymidylate synthetase. The synthesis of TMP catalyzed by thymidylate synthetase involves the transfer of a 1-carbon unit from 5, 10-methylenetetrahydrofolate acid and the loss of the C-6 hydrogen from the cofactor to the methyl group of TMP (2). The dihydrofolate acid thus formed must be reduced by dihydrofolate reductase to tetrahydrofolate acid, for it is only at this level of reduction that the folate can accept 1-carbon units to form the cofactors for intracellular reactions (Chart 7). Interruption of the dihydrofolate reductase reaction, as by MTX, with the continuing synthesis of TMP results in depletion of reduced folate cofactor pools and growth inhibition (24). However, this depletion of reduced folate cofactor pools would not occur in cells exposed to MTX, which contained no TMP synthetase activity, except via growth dilution. Thus, bacterial or mammalian cell mutants
when added to the medium of cells in culture, the anabobiotics, albeit at the expense of thymidylate synthesis. Other deoxynucleotides. Thus, the generation of excess MTX and thymine or dThd (25). Under these conditions no purine toxicity is expressed. Limitation of the thymidylate pool, in dThd (100 ,@M or greater) required to achieve the modulation of reduced folate cofactors, allowing other folate-requiring reactions such as purine de novo synthesis.

An integral part of this mechanism is that a partial reversal of MTX toxicity may well be achieved dependent on the balance of the 2 key enzymes in the folate oxidation-reduction cycle (Chart 7). Such a partial reversal has been observed both in cell culture (3, 11, 36) and in vivo (Chart 1). Antitumor selectivity would be achieved through the differential ability of dThd to induce an inhibition of thymidylate synthetase in various cell types. Little or no protection from MTX toxicity with the addition of dThd has been observed for most mammalian cell lines in culture (8, 9, 36). Thus, the mechanism of MTX tumor cell kill in the presence of dThd would be a limitation of purine nucleotide biosynthesis, with L1210 undergoing a "purineless" death similar to that observed in L5178Y cells in culture by Hryniuk (13). Additional support for this concept is derived from the observations that the antitumor activity obtained with the MTX-dThd combination is reversed by the administration of inosine (Table 2) and the high plasma concentration of dThd (100 ,@M or greater) required to achieve the modulation. A lower concentration of dThd, which was effective in blocking toxicity in the presence of inosine (Table 1), was ineffective alone in preventing MTX toxicity. Similar results were obtained by Pinedo et al. (28) who evaluated the ability of nucleosides to prevent the cytotoxicity of MTX to mouse bone marrow cells in vitro.

Although these results indicate that the therapeutic index of MTX in the mouse is clearly improved by coadministration of dThd, the implications of extrapolating these data to the clinic are complicated by the salvage metabolite effects themselves. In contrast to the mouse serum, human sera does not contain xanthine oxidase (1), and thus the report of 40 ,@M hypoxanthine in human sera is not surprising (26). Variation in endogenous purines and other normal metabolites may play a significant role in modulating MTX activity against human tumors. Although MTX and concurrent dThd has been initiated into clinical trial with some therapeutic success (5), clearly, further studies are necessary to evaluate the role of salvage metabolites in clinical MTX as well as other antimetabolite chemotherapies.

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