Improved Therapeutic Index with High-Dose Methotrexate: Comparison of Thymidine-Purine Rescue with Citrovorum Factor Rescue in Mice

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ABSTRACT

Two biochemically different rescue agents, citrovorum factor (CF) and thymidine-inosine-allopurinol (TIA), were compared in an attempt to identify the mechanism for the increased therapeutic index achieved with high-dose methotrexate (MTX) plus rescue.

Both CF and TIA were capable of protecting mice from MTX dosages up to 2000 mg/kg. Treatment of L1210-bearing mice with 2000 mg/kg MTX plus CF or TIA produced a 70 and 100% increase in life span, respectively, compared with 29% increase in life span achieved with the maximally tolerated dose of MTX alone. Bioassay of surviving peritoneal L1210 cells showed that a 4.5-log tumor kill occurred 24 hr after 2000 mg/kg MTX, while 400 mg/kg MTX produced only a 2-log cell kill. This differential tumor kill in the 24-hr period after MTX and prior to the onset of rescue accounted for the observed increase in animal survival times. In addition, treatment with 2000 mg/kg MTX resulted in a one-log-greater tumor kill of cells metastasized to the brain than did treatment with 400 mg/kg MTX.

Following 2000 mg/kg MTX, additional tumor kill, as measured by bioassay, occurred during the period of TIA rescue but not during CF rescue, which was consistent with the observed differences in survival times between CF- and TIA-rescued mice. DNA synthesis in tumor and host tissue, as measured by the rate of \( ^{3}H \)dCyd incorporation into DNA, was cyclic after TIA administration but not after CF administration. The cyclic nature of DNA recovery in TIA-treated mice paralleled plasma kinetics of thymidine. It is postulated that "thymineless" intervals created by the rapid disappearance of thymidine resulted in inhibition of DNA synthesis and additional tumor cell kill during TIA rescue. Normal tissue did not appear to be adversely affected by exposure to these "thymineless" intervals.

INTRODUCTION

The antimitabolite MTX, like most anticancer drugs, has limited selectivity and, as a result, its effectiveness is limited by toxicity to normal proliferating cells, particularly intestinal epithelium and bone marrow. One approach towards improving the therapeutic index of MTX is to administer potentially lethal doses of the drug, followed by a "rescue" agent to prevent serious toxicity to normal cells (1). MTX is thought to produce its antitumor effects by inhibiting the enzyme dihydrofolate reductase (EC 1.5.1.3.) and depleting intracellular levels of reduced folate cofactors required for de novo purine, thymidylate, and protein synthesis (15). The metabolic block produced by MTX can be circumvented by providing either a reduced folate (i.e., CF) or the end products of folate metabolism, namely, dThd and a purine source. Goldin et al. (6-8) first demonstrated that simultaneous or delayed administration of CF could protect normal mice from toxic doses of MTX. These same investigators showed that delayed administration of CF in tumor-bearing animals could prevent MTX-induced host toxicity without diminishing the antitumor activity of the drug (6-8). Since the original observations of Goldin et al., high-dose MTX plus CF rescue has been used with some success in the treatment of certain human cancers (4, 13, 14). Despite many studies, however, the mechanism by which CF enhances the therapeutic index of MTX is not well understood.

Protection of cells from MTX toxicity using dThd and a purine source was first shown in vitro by Hakala et al. (9, 10). Studies by Straw et al. (23) show that MTX toxicity can be prevented in mice by administration of hypoxanthine:allopurinol:dThd. In tumor-bearing animals, Harrap et al. (11) demonstrated an improved antitumor activity when MTX was followed by hypoxanthine:allopurinol:dThd rescue.

Despite differences in their respective biochemical and pharmacokinetic properties, both CF and dThd:purine are capable of increasing the therapeutic index of MTX when given in the appropriate dose-schedule. This ability of both CF and dThd:purine to enhance the antitumor activity of MTX suggests that a factor common to both rescue agents may be responsible for this effect. An understanding of this common factor would help to elucidate the basis for the selectivity of high-dose MTX with rescue.

In the present studies, we confirm the ability of CF or TIA to enhance the therapeutic efficacy of MTX in L1210-bearing mice. The findings suggest that cell kinetic differences between host and neoplastic cells are responsible for the selective actions of high-dose MTX plus rescue. In addition, we observed that nucleoside rescue is superior to rescue with CF in terms of increasing the survival times of tumor-bearing mice. The basis for this improved therapeutic efficacy of MTX using nucleoside rescue is discussed.

MATERIALS AND METHODS

Chromes. MTX, CF, and dThd were provided by Dr. V. Narayana, National Cancer Institute (Bethesda, MD). Inosine, thymine, and dCyd were purchased from Sigma Chemical Co. (St. Louis, MO). \( ^{3}H \)dCyd (28.3 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Allopurinol was a gift from Burroughs-Wellcome Laboratories (Research 2278 CANCER RESEARCH VOL. 44

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4 The abbreviations used are: MTX, methotrexate; CF, citrovorum factor; dThd, thymidine; ILS, increase in life span; TIA, thymidine-inosine-allopurinol; dCyd, deoxy-cytidine; PCA, perchloric acid; t.i.d., 3 times/day.

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Triangle Park, NC). All other chemicals were of reagent grade, except methanol, which was high-pressure liquid chromatography grade.

Animals. Male C57BL/6 × DBA/2 (hereafter called BDF2) mice purchased from Sprague-Dawley Laboratories (Madison, WI) were used in all experiments. Mice ranged in weight from 22 to 25 g. In the bioassay studies, male DBA/2 mice (20 to 24 g) were used as recipient mice. Animals were fed standard lab chow and water ad libitum. L1210 ascites cells were maintained in the peritoneal cavity of DBA/2 mice by weekly passage of 10^7 cells.

Survival Studies. BDF2 mice were treated i.p. with MTX (dissolved in 2% to 4% NaHCO3) on Day 4 after inoculation i.p. with 10^7 L1210 cells. At 24 hr after MTX, mice were given p.i. injections of either CF (6 mg/kg) or TIA (dThd, 500 mg/kg; inosine, 50 mg/kg; allopurinol, 10 mg/kg) t.i.d. for 5 consecutive days. (Allopurinol, a xanthine oxidase inhibitor, was included to block purine catabolism.) Unrescued controls received 0.9% NaCl solution (saline).

Preliminary studies showed that endogenous pools were saturated by a dCyd dose of 50 mg/kg, and incorporation of [3H]dCyd was linear for 30 min (data not shown).

Mice were killed by cervical dislocation 30 min after inoculation with [3H]dCyd (2 mCi/kg; 50 mg/kg), and L1210 cells were flushed from the peritoneal cavity with 0.9% NaCl solution. CF was used to accelerate the loss of MTX from the tissue. The cells were centrifuged, and the pellet was resuspended in 5 ml of hypotonic saline (0.2% NaCl solution) to lyse RBC and returned to isotonicity with 1 ml of 4.5% NaCl solution. After the supernatant was discarded, the cell pellet was suspended in 5 ml of 0.2 n PCA and stored at 4°C overnight.

Small intestine was obtained by cutting 1 to 2 cm distal to the pylorus and removing 18 cm of intestine. Fatty tissue was removed, and the lumen was flushed clean with saline. The tissue was homogenized in 6 ml of 0.2 n PCA, and the homogenate was stored at 4°C until further analysis.

The femur from each mouse was removed, the ball joint was clipped, and the marrow was flushed from the femur with 1.5 ml of 0.2 n PCA. The marrow from two femurs was pooled, and the samples were stored at 4°C.

DNA from the tissue samples was extracted by a modified Schmidt-Thannhauser procedure (23). All samples were centrifuged at 1000 × g for 5 min. The precipitated material was suspended in 4 ml of 0.2 n PCA, centrifuged at 2000 × g for 10 min, and then washed twice with 4 ml of 0.2 n PCA (bone marrow, tumor) or 8 ml of 0.2 n PCA (small intestine) in order to remove acid-insoluble materials. Lipids were extracted once (bone marrow, tumor) with 4 ml of ethanol:ether (3:1, v/v) or twice (small intestine) with 8 ml of ethanol:ether. After each washing, the pellets were centrifuged at 500 × g for 5 min. RNA was hydrolyzed by suspending the precipitate in 4 ml of 0.5 n KOH and heating in a water bath for 1 hr at 37°C. Following incubation, 1.2 ml of 50% trichloroacetic acid was added to give a final concentration of 5% trichloroacetic acid, and the sample was chilled for 20 min. The DNA pellet was washed once with 4 ml of 0.2 n PCA, resuspended in 0.5 n PCA, and hydrolyzed for 20 min at 90°C. The samples were centrifuged, and the DNA-containing supernatant was saved for determination of radioactivity and DNA concentration.

Duplicate 500-μl aliquots of the supernatant were analyzed by the method of Burton (3) to determine DNA concentration. Radioactivity was measured using liquid scintillation spectrometry.

Determination of the Number of Nucleated Bone Marrow Cells. The femurs of sacrificed mice were dissected from the mice. The marrow was flushed twice with 2.5 ml of Isoton II (Coulter Diagnostics, Hialeah, FL), and the marrow from two femurs was pooled. Three drops of Zapsilon (Coulter Diagnostics) were added to lyse the membranes of nucleated and nonnucleated cells. After the cell suspension was gently mixed, nuclei were counted electronically using a Model 2F Coulter Counter (100-μm aperture tube).

Separation and Quantitation of dThd, Thymine, Inosine, and Hypoxanthine. Blood was obtained from anesthetized mice from the orbital sinus following removal of the eye. The blood was collected in 1.5 ml microcentrifuge tubes. Plasma was diluted 1:1 with distilled water and deproteinized by boiling for 15 min at 90°C and with 1 n PCA. The supernatant was neutralized with an appropriate volume of 2 n KOH and stored at −6°C until further analysis.

Purine and pyrimidine nucleosides and bases were separated by a high-pressure liquid chromatography method modified from the method of Rustum (17). The solvent delivery system consisted of a Waters M-45 and 6000A pump controlled by a Model 660 solvent programmer (Waters Associates, Milford, MA). Samples were injected onto a C-18 reverse-phase Column (LiChrosorb RP 18, 10 μm; Altex Scientific, Inc., Berkeley, CA) and eluted with 5% methanol in water. Samples were detected using a Model 440 UV absorbance detector (254 nm; Waters Associates). Peak areas were integrated using either a Hewlett-Packard 3381 A Integrator (Hewlett-Packard, Corvallis, OR) or a Waters Data Module (Water Associates), and concentrations were calculated based on the external standard method.

Bioassay Method for Quantitating L1210 Cells. A bioassay technique, based on the procedure of Skipper et al. (22), was used to quantitate the number of L1210 cells which survive MTX treatment. The advantage of the bioassay compared with dye exclusion and electronic counting is that the bioassay measures only tumor cells which retain the ability to proliferate and ultimately kill the host.

Mice were inoculated i.p. with 10^7 L1210 cells and treated on Day 4 with either saline (untreated controls), MTX (400 mg/kg), or MTX (2000 mg/kg). At 24 hr after MTX, mice were sacrificed, and tumor cells were flushed from the peritoneal cavity with 0.1 mM CF in 0.9% NaCl solution (saline).

The number of surviving animals was determined daily, and median survival time of each treatment group was calculated as the day after tumor inoculation, when the number of surviving animals was (N − 1)/2. Data from several survival experiments were pooled, and statistical analysis of the median survival times was carried out using the non-parametric Mann-Whitney U test.

Tumor-bearing and non-tumor-bearing mice were weighed daily, and removing 18 cm of intestine. Fatty tissue was removed, and the lumen was flushed clean with saline. The tissue was homogenized in 6 ml of 0.2 n PCA, and the homogenate was stored at 4°C until further analysis.

The femur from each mouse was removed, the ball joint was clipped, and the marrow was flushed from the femur with 1.5 ml of 0.2 n PCA. The marrow from two femurs was pooled, and the samples were stored at 4°C.

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RESULTS

Protection from MTX Toxicity. Animals treated with a single dose of MTX (400 mg/kg) lost 25% of their body weight (Chart 1) and died within 6 days. However, when either CF or TIA was administered, serious weight loss and animal deaths were prevented. These results (Chart 1) demonstrate that CF and TIA could each protect mice from drug toxicity following MTX doses as high as 2000 mg/kg. An equimolar concentration of thymine (260 mg/kg) was found to substitute for dThd in the TIA rescue regimen and protect mice from MTX toxicity. However, dThd alone (500 mg/kg) given t.i.d. for 5 days failed to prevent animal deaths after a single injection of MTX (2000 mg/kg) (results not shown).

The effect of CF and TIA on reversing MTX toxicity to bone marrow was determined in leukemic mice treated with MTX followed by either CF or TIA rescue. In untreated control mice, there were 2.03 x 10^7 nucleated cells/femur (Chart 2). Treatment with MTX (400 mg/kg) and MTX (2000 mg/kg) produced a nadir of 9.6 and 4.6 x 10^6 cells, respectively. When CF or TIA was given after MTX (2000 mg/kg), the total number of nucleated marrow cells at the nadir was slightly greater than that in unrescued animals. Recovery following MTX plus CF occurred after 4 days, whereas no apparent recovery of marrow cells was observed by Day 6 in TIA-treated animals. However, recovery of bone marrow cells was sufficient to prevent host lethality, since the mice given TIA rescue survived potentially lethal doses of MTX.

High-Dose versus Conventional-Dose MTX. Animals were treated with various doses of MTX 4 days after inoculation with 10^6 L1210 cells. Beginning 24 hr after MTX, animals were given either no rescue, CF, or TIA t.i.d. for 5 days. The maximum dose of MTX alone which caused no toxic deaths was 200 mg/kg. As described earlier (Chart 1), animals could tolerate MTX dosages up to 2000 mg/kg without serious toxicity when CF or TIA rescue was given. As seen in Chart 3, a dose-dependent increase in life span occurred which appeared to plateau between 400 and 1000 mg/kg, and which then dramatically increased following MTX (2000 mg/kg). The percentage of ILS achieved with the maximum dose of MTX (2000 mg/kg) with rescue was significantly greater (p < 0.001) than that achieved with the maximum dose of MTX alone (200 mg/kg). Furthermore, TIA rescue was superior to rescue with CF. In mice given MTX (2000 mg/kg), rescue with CF and TIA resulted in a 70 and 100% ILS, respectively (p < 0.001). The difference between CF and TIA rescue was not apparent at MTX doses of less than 2000 mg/kg.

Quantitation of L1210 Cell Kill. Chart 4 shows the number of i.p. clonogenic cells present at various times after MTX administration. Twenty-four hr after MTX and prior to the start of rescue, MTX at 400 mg/kg produced about a 2-log tumor cell kill, whereas the 2000-mg/kg dose produced about a 4.5-log tumor cell kill. Based on the tumor burden present at 24 hr and a doubling time of 13 hr, the day of death (when tumor burden reached 1.5 x 10^6 cell) was predicted to be 8.5 days for control, 13 days for MTX (400 mg/kg), and 17 days for MTX (2000 mg/kg). The observed survival times were 7.8 days for control, 9.6 days for MTX (400 mg/kg) plus rescue with CF, and 9.9 days for MTX (400 mg/kg) plus rescue with TIA. After MTX (2000 mg/kg), mice survived for 13.4 days when rescued with CF and 16.8 days when rescued with TIA. These data show that the major...
way in which the high-dose regimen increased survival time was by producing a greater tumor cell kill in the 24 hr prior to rescue.

Chart 4 also shows that resumption of tumor cell growth occurred in both rescued and unrescued animals. During the first 48 to 72 hr of rescue, the apparent doubling times in CF- and TIA-rescued mice were 15 and 36 hr, respectively. After Day 8, the rate of tumor regrowth was similar in both CF- and TIA-rescued animals, and it paralleled the growth rate in untreated mice. Delayed resumption of growth in the TIA-rescued mice resulted in a lower number of clonogenic cells at Day 10 as compared to the CF-rescued mice. Furthermore, this difference in tumor burden at Day 10 was sufficient to account for the difference in survival times (13.5 days with CF rescue and 16.8 days with TIA rescue).

**Effect of MTX Dose on L1210 Cells That Metastasized to the Brain.** A bioassay was used to test if a relationship exists between the dose-related increase in tumor kill and the capacity to eradicate L1210 cells which migrate to the brain. Mice were treated on Day 4 after i.p. inoculations of 10⁶ L1210 cells with MTX at either 400 or 2000 mg/kg. Twenty-four hr after MTX, mice were sacrificed, and the number of clonogenic tumor cells was determined by bioassay. There was an approximately one-log difference in tumor kill between mice given MTX (400 mg/kg) and mice given MTX (2000 mg/kg) (data not shown). Since the growth kinetics of L1210 cells in the brain is the same as in the peritoneal cavity and since there is a specific tumor burden associated with animal death (22), this 1-log difference corresponds to a 1.5-day increase in animal survival time. This differential tumor kill of L1210 cells which have metastasized to the brain appears to account for only a portion of the total (4 to 7 days) increase in median survival times between animals given MTX (400 mg/kg) and those given MTX (2000 mg/kg).

**Effects of High-Dose MTX plus Rescue on DNA Synthesis.** Incorporation of [³H]dCyd into DNA was used to calculate the rate of DNA synthesis in small intestine, bone marrow, and L1210 cells. In the absence of rescue, MTX inhibited DNA synthesis in all three tissues for at least 68 hr (Charts 5 and 6). Recovery of DNA synthesis was dependent on the rescue agent used. During TIA rescue, a marked resumption of DNA synthesis was observed in bone marrow and small intestine. As seen in Chart 5, the rate of [³H]dCyd incorporation into bone marrow and intestinal epithelial DNA was cyclic, peaking 2 hr after each TIA injection and returning to prerescue rates within 4 hr. As a result, intervals between TIA injections existed during which DNA synthesis remained inhibited. Although the magnitude of recovery of L1210 synthesis was less than that seen in intestinal epithelium and bone marrow, the pattern of resumption of L1210 cells was also cyclic from 24 to 48 hr.

In contrast to the cyclic recovery of DNA synthesis following TIA administration, there was a gradual resumption of DNA synthesis in small intestine and bone marrow following CF (Chart 6). During the first 2 days of rescue (24 to 48 hr after MTX), the rate of gut DNA synthesis increased from 25% of control (at 24
hr) to 50% of control (at 57 hr). During Day 3 of rescue (72 to 88 hr), the rate of DNA synthesis overshot control levels. DNA synthesis in bone marrow showed little recovery until the end of the second day (62 hr) of CF rescue. Unlike the case with the small intestine, the degree of recovery of synthesis of bone marrow DNA failed to reach 100% of control during 3 days of rescue. Little resumption of DNA synthesis was observed in L1210 cells. L1210 DNA synthesis was 10% of control 24 hr after MTX, and it remained less than 10% of control during 3 days of CF rescue.

**Plasma Kinetics of TIA.** In order to determine if the kinetics of TIA could account for the cyclic resumption of DNA synthesis, plasma levels of dThd and inosine and their metabolites thymine and hypoxanthine were determined using high-pressure liquid chromatography. Following TIA, dThd plasma levels increased 100-fold within 10 min and then declined, with an apparent half-life of 16 min (Chart 7). Plasma concentrations of dThd returned to endogenous levels 4 to 5 hr after TIA administration. Thymine, a major metabolite of dThd, increased 10-fold, peaking 30 min after TIA. Within 4 hr, thymine plasma concentrations also returned to endogenous levels. A 30-fold increase in inosine plasma levels was observed within 30 min of TIA administration (data not shown). Inosine declined rapidly and reached endogenous levels by 4 hr. Consistent with previous studies showing catabolism of inosine to hypoxanthine, plasma levels of hypoxanthine increased 20-fold within 5 min. Although hypoxanthine has a reported half-life of 13 min (12), in the presence of allopurinol, the rate of catabolism was noticeably decreased, such that levels remained above endogenous concentrations for almost the entire 7-hr period between TIA injections.

These data show that there were intervals during TIA rescue when unbalanced growth could occur due to lack of thymine at times when adequate purines were present.

**DISCUSSION**

The results presented in this study demonstrate that, in the same model system, an improved therapeutic index of MTX can be achieved using either CF or TIA rescue. Treatment of L1210-bearing mice with MTX (2000 mg/kg) followed either by CF or TIA resulted in an increase in life span that was significantly greater than that achieved with the maximally tolerated dose of MTX alone (Chart 3). These findings corroborate earlier studies showing that CF (6–8, 20) or dThd:purine (11, 23) can improve...
the therapeutic efficacy of MTX. More importantly, these results establish that 2 rescue agents which differ in their respective biochemical and pharmacokinetic properties can achieve the same end point, namely, an increase in the therapeutic index MTX.

Two important conclusions can be derived from this data. First, the major difference between low-dose MTX and high-dose MTX is the degree of tumor cell kill prior to rescue. As seen in Chart 4, treatment with MTX (2000 mg/kg) produced approximately a 2.5-log greater tumor kill than did treatment with MTX (400 mg/kg) during the 24-hr period following MTX, when virtually all tumor kill occurred. Second, this increase in tumor kill occurred without a proportional increase in host toxicity. No significant toxic effects on animal body weight or survival were seen in normal mice given potentially lethal doses of MTX followed by either CF or TIA rescue. This suggests that, while tumor cells are sensitive to increased levels of MTX, host tissue can tolerate larger MTX doses if rescue is begun within 24 hr of MTX treatment.

The prolonged survival times achieved with high-dose MTX may result in part from an increased ability to kill metastasizing cells, particularly cells migrating to the brain. Since most tumor-bearing hosts die as a consequence of disseminated disease (and the brain often serves as a protected sanctuary for tumor cells), eradication of metastasizing tumor can significantly affect the host's response to chemotherapy. One disadvantage of MTX is that, like other polar compounds, it has a reduced ability to penetrate the blood-brain barrier and achieve sufficient concentrations to kill tumor cells in the brain. Results from this study show that a single i.p. injection of MTX (2000 mg/kg) produced approximately a 1-log greater tumor kill of cells in the brain than did treatment with MTX (400 mg/kg).

High-dose MTX may have pharmacological actions different from low-dose MTX which could explain the differential tumor cell kill observed between MTX (400 mg/kg) and MTX (2000 mg/kg). Assuming that survival times reflect the extent of tumor cell kill, the dose-response survival curve illustrated in Chart 3 shows a plateau for tumor kill between MTX (400 mg/kg) and MTX (1000 mg/kg), after which an abrupt increase in life span occurs with MTX (2000 mg/kg). This dose-response relationship is uncharacteristic for a self-limiting, phase-specific drug like MTX (2), with which increasing drug doses produce greater tumor kill until a plateau is reached, after which no additional kill occurs. Also, since 65 to 75% of L1210 cells are in S phase (21), the greater than 99.99% tumor kill following MTX (2000 mg/kg) (Chart 4) suggests that either high-dose MTX is not a self-limiting agent or it can kill cells in other parts of the cell cycle. Although the precise mechanism for the enhanced tumor kill remains to be determined, results from this study suggest that high-dose MTX has sites of action additional to those of lower-dose MTX.

The observed increases in tumor cell kill following MTX (2000 mg/kg) were not accompanied by a proportional increase in toxicity to normal cells. The increase in therapeutic index of high-dose MTX rescue may result from cell kinetic differences between normal and malignant cells, namely, the presence of a normal stem cell population. Pinedo et al. (16) demonstrated that stem cell recruitment occurs in the presence of MTX levels which inhibit RNA and DNA synthesis, indicating that RNA and DNA are not required for recruitment in the cell cycle. Thus, increases in MTX concentration would not necessarily increase the depletion of the normal stem cell population. Administration of rescue 24 hr after MTX reverses host toxicity, suggesting that the stem cell reserve was not depleted prior to rescue intervention.

Survival times of leukemic mice given MTX (2000 mg/kg) were significantly different, depending on whether CF (70% ILS) or TIA (100% ILS) rescue was administered. The different effects of CF and TIA on tumor kill are illustrated in Chart 4, and they show that the number of surviving L1210 cells in the peritoneal cavity of MTX CF-treated mice was greater than that in mice given MTX/TIA. Using data on tumor burden that were generated from the bioassay experiments (Chart 4), the survival times of treated mice were calculated and were found to corroborate observed survival times. Harrap et al. (11), using a dose schedule similar to that used in this lab, have also reported that TIA or HAT rescue was superior to CF rescue in terms of prolonging survival times of leukemic mice. However, no explanation for this observation was offered by these investigators.

Results from this study suggest that tumor cell kill can occur during TIA rescue but not during CF rescue and that it accounts for differences between CF and TIA on leukemic animal survival. We postulate that, during TIA rescue, periodic intervals occur during which tumor cells, which were not killed prior to rescue, can undergo thymineless death, a condition of unbalanced cell growth which occurs when DNA synthesis is blocked yet RNA and protein synthesis remain undisturbed (5). If, however, RNA and protein synthesis are inhibited along with DNA, the cell does not die but enters a quiescent state and is insensitive to phase- or cycle-specific agents such as MTX. Cells can be forced into the cell cycle again if RNA and protein synthesis are restored. In support of this hypothesis, we found that: (a) recovery of MTX-inhibited DNA synthesis was cyclic following TIA administration (Chart 5), with periods between TIA injections when DNA synthesis was blocked; (b) plasma levels of dThd paralleled the recovery of DNA synthesis, increasing after TIA and then returning to endogenous levels within 4 hr. During intervals between TIA injections, when dThd levels approximated endogenous levels, purine concentrations remained elevated, creating the condition for unbalanced growth; and (c) when clonogenic tumor cells were measured by bioassay, the rate of tumor regrowth during TIA rescue was reduced, suggesting that additional tumor kill occurred. In contrast, regrowth of peritoneal tumor cells during CF rescue paralleled tumor regrowth in untreated control animals.

It is unclear why normal tissue is not adversely affected by thymineless intervals during TIA rescue. Based on observation of cell kinetic parameters, it would be predicted that host cells would be subject to the toxic actions of MTX during the periodic thymineless intervals. However, any toxic effects which may occur did not significantly affect the host. This observation is important, because it does not follow a predicted pattern of response. If, indeed, normal cells are selectively spared during thymineless intervals, this would have important implications in the design of future rescue protocols.

The findings presented in this study demonstrate that the increase in therapeutic index of MTX results from an enhanced tumor kill prior to rescue and requires a dose of MTX above a threshold level. In addition, nucleoside rescue had some therapeutic advantage over CF when given at periodic intervals. This observation may have clinical implications; however, caution using nucleoside rescue in humans must be followed because of potential toxicities. Although no serious toxicities were observed in animals rescued with TIA, we did find that recovery of
nucleated bone marrow cell growth (Chart 2) was delayed, and this could be significant when the limiting toxicity is bone marrow, as it is in humans.

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