Biochemical Pharmacology, Vol. 30. No. 8, pp. 869–874, 1981.
Printed in Great Britain.

0006-2952/81/080869-06 $02.00/00

MODIFICATION OF *IN VIVO* METHOTREXATE ANTITUMOR EFFECT IN L1210 LEUKEMIA BY INDUCED IMPAIRMENT OF PURINE SALVAGE*

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(*Received* 9 *June* 1980; *accepted* 29 *September* 1980)

Abstract—Studies with murine cells have shown that the antitumor action of methotrexate (MTX) may be through a purineless mechanism. If the MTX effect depends, in part, on inhibition of *de novo* purine synthesis, then the ability of tumor cells to salvage available purine precursors could reduce the cell kill. In the present study, we produced L1210 murine leukemia mutants with impaired purine salvage to determine whether this would affect responsiveness to MTX. Mutant lines L1210/MP, L1210/FAMP, and L1210/555 were produced by developing resistance to the purine analogs 6-mercaptopurine (6-MP), 6-MP + 2-fluoroadenine (2-FA), and 6-MP + 2-FA + 6-methylmercaptopurine riboside respectively. The purine salvage capability of the cell lines was confirmed *in vitro* by testing the ability of various purines to reverse the growth inhibitory and biochemical effects of MTX in the presence of thymidine. Dose–response curves demonstrated identical *in vitro* MTX sensitivity for L1210/MP, L1210/FAMP, and the parent line, L1210/S. Despite identical *in vitro* MTX sensitivity, the cell lines L1210/MP and L1210/FAMP displayed increased sensitivity to the biochemical effects of MTX in an *in vivo* model, and this was translated into enhanced sensitivity as measured by survival experiments in tumor-bearing mice. The results indicate that impairment of purine salvage sensitizes cells to the antitumor effect of MTX *in vivo*. This has implications for the clinical use of MTX in view of the variety of rescue techniques that is available.

Clinical studies have confirmed that the administration of thymidine can protect patients from methotrexate (MTX)† toxicity [1, 2]. Since MTX inhibits *de novo* purine biosynthesis, as well as thymidylate synthesis, prevention of MTX toxicity presumably depends upon the availability of salvageable purines. Purine salvage is the primary route used by bone marrow cells for nucleic acid synthesis in some mammalian species [3]. In addition to modifying the toxic effect of MTX, purines may also influence the antitumor effect of the drug as suggested by the observation that allopurinol decreased the therapeutic effectiveness of MTX in L1210 tumor-bearing mice [4]. Clarification of the role of purines in modulating the effects of antimetabolites would be helpful in the design of chemotherapeutic strategies, especially in circumstances where a variety of "rescue" techniques is available [5].

It has already been demonstrated in experimental L1210 leukemia that the use of purine and thymidine rescue from potentially lethal doses of MTX can provide a therapeutic effect superior to that achieved when folinic acid is used as the rescuing agent [6]. MTX has been shown to produce cell lethality through a purineless effect in murine tumor cell lines [7]. It is postulated that cells relying on *de novo* purine biosynthesis may be more susceptible to the purineless effect of MTX, resulting in enhanced *in vivo* MTX sensitivity. The purpose of these studies was to test this hypothesis.

MATERIALS AND METHODS

L1210 murine leukemia cells were obtained originally from the Arthur D. Little Co. (Cambridge, MA). Male DBA_2 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and used when they were 8–12 weeks of age. 6-MP and MeMPR as well as glutamine, PEI cellulose, Tris buffer, and nucleosides and bases were purchased from the Sigma Chemical Co. (St. Louis, MO). 2-FA was a gift from Ash Stevens Inc. (Detroit, MI). MTX for *in vitro* use and citrovorum factor were purchased from Cyanamid of Canada, Inc. (Montreal, Quebec). For *in vivo* use, MTX (88.8 per cent purity) was supplied as a gift from Dr. Richard Lacombe, Cyanamid of Canada, and was solubilized in sodium hydroxide and brought to physiologic pH prior to injection. [5-^{3}H]CdR (sp. act. 28.3 Ci/mmole), [2,8-^{3}H]adenine (24.5 Ci/mmole), and [5-^{3}H]hypoxanthine (6.0

* Supported by Ontario Cancer Treatment and Research Foundation Project 366, and by the National Cancer Institute of Canada.

† Abbreviations used are: MTX, methotrexate; 6-MP, 6-mercaptopurine; MeMPR, 6-methylmercaptopurine riboside; PEI, polyethyleneimine; 2-FA, 2-fluoroadenine; [5-^{3}H]CdR, tritiated deoxycytidine; MEM, minimum essential medium; FBS, fetal bovine serum; PRPP, phosphoribosylpyrophosphate; APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine-phosphoribosyltransferase; TCA, trichloroacetic acid; CF, citrovorum factor; TdR, thymidine; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Ci/mmole), as well as omnifluor, were purchased from the New England Nuclear Corp. (Boston, MA). Eagle's MEM supplemented with Earle's salts and nonessential amino acids, and Linbro multiwell plates were purchased from Flow Laboratories (Mississauga, Ontario). HEPES buffer and FBS were purchased from Grand Island Biologicals (Grand Island, NY). Flasks for tissue culture and polypropylene incubation tubes (12 × 75 mm) were purchased from Falcon Plastics (Oxnard, CA). Eppendorf polypropylene microcentrifuge tubes (1.5 ml capacity) were purchased from Brinkmann Instruments Inc. (Westbury, NY). 2-Mercaptoethanol was purchased from the Eastman Kodak Co. (Rochester, NY). PRPP was purchased from Boehringer Mannheim and stored at 4°. Sephadex G25 was purchased from Pharmacia (Canada) (Dorval, Quebec). Whatman glass fibre discs (GF/C) for filtration were purchased from CANLAB (Toronto, Ontario). All routine chemicals were analytical grade.

Derivation of mutant cell lines. Murine L1210 cells were maintained in logarithmic growth in suspension in modified Eagle's MEM supplemented with 10% FBS, 20 mM HEPES buffer, 50 μM 2-mercaptoethanol, 0.03% L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) in an atmosphere of humidified air plus 4% CO_2. Mutant cell lines were developed by producing resistance to appropriate base and nucleoside analogs. Resistance to 2-FA was accomplished *in vitro* by exposure of parent cells (L1210/S) and 6-MP resistant cells (L1210/MP) to gradually increasing concentrations of this compound until cells attained control growth rate in the presence of 1 μM and 10 μM 2-FA to produce cell lines L1210/FA and L1210/FAMP respectively.

Resistance to 6-MP was produced *in vivo* by i.p. injection of drug (60 mg/kg) daily for five successive days through five serial passages in DBA_2 mice bearing L1210/S ascitic tumors. Resistance was verified *in vitro*, using dose–response curves, and *in vivo* by assessing the effect of drug treatment on the survival of tumor-bearing mice (see Results).

Resistance to MeMPR was produced *in vivo* in the same manner as described by 6-MP using a dose schedule of 30 mg/kg daily for three successive days over four passages; resistance was confirmed by *in vitro* dose–response curves.

Multiple resistant cell lines were produced by sequential exposure to the appropriate drugs in the manner described and in the following sequence:

$$\text{L1210/S} \xrightarrow{\text{6-MP}} \text{L1210/MP} \xrightarrow{\text{2-FA}} \text{L1210/FAMP}$$
$$\xrightarrow{\text{MeMPR}} \text{L1210/555}$$

Dose–response experiments. Dose–response experiments were carried out to confirm resistance and to test for sensitivity to MTX. They were always performed in medium supplemented with dialyzed serum. Cells were maintained in drug-free medium for 24–48 hr prior to testing. The concentration of drug that inhibited growth to 50 per cent of that of control cultures after 48 hr of incubation (IC_{50}) was used as the standard expression of drug sensitivity, and the ratio of the IC_{50} of the mutant, resistant cells (L1210/R) to the IC_{50} of the L1210/S cells was used to express drug sensitivity of a cell line relative to the parent control cells. Absolute values of IC_{50} concentrations are not sufficiently reproducible to make accurate comparisons of a number of experiments performed over a prolonged time interval; therefore, when dose–response experiments were performed, the control cell line was tested in parallel with each mutant line in each separate experiment, and the ratio of the IC_{50} of the test cell line to the control, for MTX, was calculated. This expression of relative drug resistance has been found to give a reproducible value [8]. All cell lines were tested for mycoplasma infection and were negative.

Enzyme assays. Enzymes were assayed to confirm the mechanisms of 2-FA and 6-MP resistance in cell lines L1210/MP, L1210/FAMP, and L1210/555. APRT and HGPRT were assayed by the method of Schmidt *et al.* [9]. Extracts obtained from sonicated cells were passed through a Sephadex G25 column using 50 mM Tris–HCl (pH 7.5) as eluent. A volume of eluate (containing about 50 μg protein) was added to the reaction mixture containing 200 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM PRPP, and labeled substrate ([^{3}H]adenine or [^{3}H]hypoxanthine, 10 μCi/ml) to a total volume of 350 μl. Cold adenine or hypoxanthine was included to a final concentration of 40 μM. The reaction proceeded at 25° and, at intervals, aliquots were removed and added to 100 vol. of 1 mM ammonium acetate (pH 5) containing 100 μM adenine or hypoxanthine. Samples were filtered through GF/C discs treated previously with PEI cellulose. The discs were washed with 5 ml ammonium acetate (pH 5) and then dried and counted. Enzyme activity was calculated from the slopes of the reaction lines generated which were linear over 24 min. Protein was determined by the method of Lowry *et al.* [10].

Assessing purine salvage properties in vitro. All "salvage" experiments were performed using medium supplemented with dialyzed serum, and cells were maintained in drug-free medium for 24 hr prior to testing.

Purine salvage was measured *in vitro* by assessing the abilities of various purine bases and nucleosides to prevent MTX-induced inhibition of cell growth or incorporation of [5-^{3}H]CdR into acid precipitable material. For growth experiments, triplicate cell cultures at 10^5/ml were exposed for 48 hr to 0.1 μM MTX and 25 μM thymidine in the presence or absence of 25 μM purines. At the end of the incubation, cells were electronically counted (Coulter model ZF); the cell concentration in treated cultures was expressed as a percentage of that in control cultures unexposed to drug or exposed to purines and thymidine in the absence of MTX.

For [5-^{3}H]CdR incorporation, 5×10^5 cells/ml were incubated with 1.0 μM MTX and 10 μM thymidine, in the absence or presence of purine precursors at a final concentration of 10 μM. After 3 hr, cells were pulsed with [5-^{3}H]CdR (2 μCi/ml), and aliquots were removed at intervals over 20 min and precipitated with ice-cold 10% TCA. Samples were filtered onto GF/C filter discs, washed twice with cold 10% TCA and once with 95% ethanol, and then heat-dried and counted in a Beckman LS-230. The

rates of incorporation were plotted as cpm over time. The slopes of the incorporation curves were calculated, and the results were expressed as the ratios of MTX-treated cultures to untreated controls. The ratios were then converted to per cent of control. Results of control experiments were used to compare relative MTX sensitivity.

[5-^{3}H]*CdR incorporation into acid precipitable material of ascitic* L1210 *tumors harvested from treated animals.* Tumor cells (5×10^5) were implanted i.p. into DBA_2 mice 3 days prior to MTX therapy. Test animals received MTX (250 mg/kg or 400 mg/kg s.c.) while control animals received 0.9% NaCl solution. Five and one half hours after MTX, animals received "rescuing" agents s.c.—TdR (250 mg/kg) alone, or TdR (250 mg/kg) + Hx (25 mg/kg)—while controls received 0.9% NaCl solution. One-half hour following rescue (6 hr following MTX), cells were harvested from the peritoneal cavity by lavage with 0.5 ml of unsupplemented warm Eagle's medium. The cells from three animals were pooled, and the volume was brought to 1 ml with unsupplemented Eagle's medium and kept at 37°. An aliquot of cells was electronically counted and a separate aliquot was pulsed with [5-^{3}H]CdR to a final specific activity of 10 μCi/ml. Cold CdR was included in the incubation to a final concentration of 10 μM. At appropriate intervals, aliquots of cells were removed, precipitated with cold TCA, and processed as described for *in vitro* incorporation studies. Counts were adjusted for cell numbers, and the rate of incorporation was calculated from the slope of the incorporation curve. The effects of MTX and rescuing agents are expressed as a percentage of the slope of [5-^{3}H]CdR incorporation obtained in controls.

Survival studies. Survival experiments were conducted with male DBA_2 tumor-bearing mice according to methods described previously [6]. Test animals, 8–12 weeks of age, were treated with a single, potentially lethal dose of MTX (600 mg/kg, i.p.) 3 days after i.p. implantation of tumor cells (L1210/S or L1210/MP or L1210/FAMP or L1210/555). From 4 to 24 hr following MTX, rescue therapy was initiated in groups of ten mice each. Mice were "rescued" with a schedule known as TIA [thymidine (500 mg/kg), inosine (100 mg/kg), and allopurinol (10 mg/kg)] given i.p. three times daily for 6 days or with CF (15 mg/kg) i.p. three times daily for 6 days. Control groups received either no therapy or MTX without rescue, so that the adequacy of MTX dose and the efficacy of the "rescue" schedules could be evaluated. The extension of survival of the treated groups is expressed in days.

RESULTS

Derivation of mutant cell lines. The IC_{50} value for 6-MP derived from dose–response curves in L1210/S is 1.1 μM, whereas the mutant lines (L1210/MP, L1210/FAMP, and L1210/555), exposed to 100 μM 6-MP, proliferated at 90, 88 and 94 per cent of their respective controls unexposed to drug. The IC_{50} value for 2-FA is 0.21 μM, whereas cell lines L1210/FAMP and L1210/555, exposed to 10 μM 2-FA, proliferated to 91 and 86 per cent of their respective controls. The IC_{50} value for MeMPR is 0.12 μM, whereas the mutant cell line L1210/555, exposed to 100 μM MeMPR, proliferated to 93 per cent of the control. The *in vitro* doubling times of L1210/S, L1210/MP, L1210/FAMP, and L1210/555 were identical (12.0 hr) when measured in mid-log phase.

Resistance of L1210/MP to 6-MP was also confirmed *in vivo* where administration of 6-MP (70 mg/kg, i.p.) daily for 5 days failed to prolong the survival of L1210/MP tumor-bearing mice, while prolonging the life span of L1210/S tumor-bearing mice by 56 per cent. The growth rate of L1210/S and L1210/MP *in vivo* was the same.

Enzyme activity of mutant cell lines. The activities of HGPRT and APRT were measured in the parent line (L1210/S) and the mutant cell lines (L1210/MP, L1210/FAMP, and L1210/555) and are expressed in pmoles of substrate converted per min per mg protein (pmoles · min^{-1} · mg^{-1}). The results (Table 1) confirm that both 6-MP and 2-FA resistance are associated with reduced activities of the salvage enzymes HGPRT and APRT respectively.

Purine salvage characteristics of mutant cell lines. Resistance to purine analogs was produced to obtain cell lines with altered purine salvage. To document this, the abilities of purines to prevent MTX cytotoxicity *in vitro* were assessed. Table 2 demonstrates how purines prevented the growth-inhibitory effect of MTX (in the presence of TdR) during a 48-hr continuous incubation. MTX alone at 0.1 μM inhibited growth to 9, 6 and 14 per cent of control cultures in L1210/S, L1210/MP, and L1210/FAMP respectively. In L1210/S, hypoxanthine and adenine completely prevented growth inhibition, whereas in L1210/MP only adenine could substantially protect from MTX inhibition, and in L1210/FAMP adenine no longer provided significant protection.

Table 1. Activity of the purine salvage enzymes APRT and HGPRT in mutant cell lines

Cell line	APRT*	HGPRT*
L1210/S	103 ± 8 (N = 3)	169 ± 21 (N = 3)
L1210/MP	65 (N = 2)	14 ± 2 (N = 3)
L1210/FAMP	Undetectable (1)	Undetectable (2)
L1210/555	Undetectable (1)	Undetectable (1)

* Activity is pmoles · min^{-1} · (mg protein)$^{-1}$ ± S.E.M.

Table 2. Protection against MTX-induced growth inhibition*

	L1210/S (% control)	L1210/MP (% control)	L1210/FAMP (% control)
MTX (10^{-7} M)	9	6	14
MTX + Hx + TdR	103	6	
MTX + A + TdR	101	51	28

* Abbreviations: MTX, methotrexate; Hx, hypoxanthine; A, adenine; and TdR, thymidine. All nucleosides and bases were present in a final concentration of 2.5×10^{-5} M.

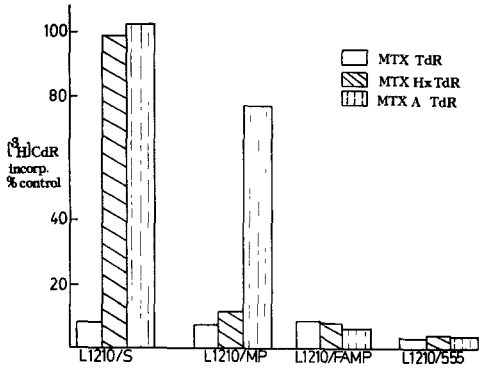

Fig. 1. Ability of purines to prevent MTX inhibition of [5-^{3}H]CdR incorporation. MTX = methotrexate (1 μM); [^{3}H]CdR = tritiated deoxycitidine (2 μCi/ml); HxTdR = hypoxanthine + thymidine (10 μM); and A TdR = adenine + thymidine (10 μM).

Purines were also tested for their abilities to prevent MTX-induced inhibition of [5-^{3}H]CdR incorporation into acid precipitable material. Figure 1 summarizes the effects in all the cell lines tested. In L1210/S, L1210/MP, L1210/FAMP and L1210/555, MTX inhibited the incorporation rate to 8.1, 7.1, 8.1 and 3.0 per cent of respective controls. Both hypoxanthine and adenine were able to prevent MTX inhibition in L1210/S, while only adenine could substantially prevent the MTX effect in L1210/MP. Neither hypoxanthine nor adenine could prevent MTX inhibition in L1210/FAMP and L1210/555.

In vitro *MTX sensitivity of mutant cell lines.* The relative *in vitro* sensitivities of mutant cell lines to MTX were determined in two ways: (1) by comparing the MTX dose response to that of L1210/S, and (2) by comparing the inhibitory effects of MTX on incorporation of [5-^{3}H]CdR into acid precipitable material of mutant versus parent cells. Table 3 summarizes the data. The IC_{50} values of MTX for L1210/MP and L1210/FAMP were similar to that for L1210/S, since the IC_{50} ratios (L1210/R/L1210/S) were close to unity. L1210/555, however, was more sensitive to MTX, since the IC_{50} ratio was significantly less than 1 (0.67).

The inhibitory effects of MTX on [5-^{3}H]CdR incorporation by L1210/MP and L1210/FAMP were also similar to that for L1210/S. Inhibition by MTX in L1210/555, however, was greater, indicating that this line was substantially more sensitive to MTX at the concentration tested. Therefore, according to both dose–response and incorporation experiments, the *in vitro* sensitivities of L1210/MP and L1210/FAMP to MTX were the same as that for L1210/S, whereas L1210/555 was more sensitive.

In vivo *metabolic effects of MTX:* [5-^{3}H]*CdR incorporation studies in cells harvested from treated animals.* Figure 2 illustrates the results of a typical *in vivo* experiment with L1210/S in which [5-^{3}H]CdR incorporation was inhibited by MTX (250 mg/kg, s.c.) to 22.8 per cent of the control value. The administration of TdR partially "restored" incorporation to 72.9 per cent of control. A summary of all the data for L1210/S and the other cell lines is presented in Fig. 3. In L1210/S, MTX inhibited incorporation to a mean of 31 per cent of control, whereas addition of TdR resulted in partial reversal to 64 per cent of control. When hypoxanthine was included with TdR, there was no inhibition by MTX, but an overshoot to 141 per cent of control. In the cell lines L1210/MP and L1210/FAMP, MTX inhibited incorporation to 12 and 11 per cent of control values respectively. In addition to the greater suppressive effect of MTX in these cell lines, TdR and TdR + hypoxanthine failed to protect from MTX.

Table 3. *In vitro* MTX sensitivity of L1210 cells: Comparison of IC_{50} values and effects on incorporation of [5-^{3}H]CdR into DNA of parent and mutant lines

	MTX IC_{50} $\frac{\text{L1210/R}}{\text{L1210/S}}$ ± S.E.M.	[5-^{3}H]CdR incorporation rate (% control) ± S.E.M.
L1210/S		8.1 ± 0.7 (N = 5)
L1210/MP	0.91 ± 0.03 (N = 5)	7.1 ± 1.6 (N = 3)
L1210/FAMP	0.97 ± 0.03 (N = 4)	8.1 (N = 2)
L1210/555	0.67 ± 0.04 (N = 3)	3.0 ± 0.1 (N = 3)

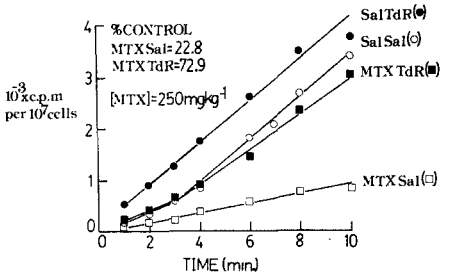

Fig. 2. *In vivo* MTX and TdR effects on [5-^{3}H]CdR incorporation rate in L1210/S tumor cells harvested from mice. MTX (250 mg/kg) was given s.c. at zero time and TdR (250 mg/kg) was given s.c. at 5.5 hr. Cells were harvested at 6 hr and pulsed with [5-^{3}H]CdR (10 μCi/ml).

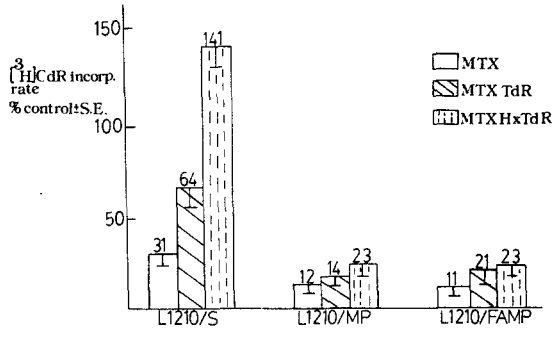

Fig. 3. Effects of MTX and TdR ± purine on incorporation of [5-^{3}H]CdR by cells harvested from mice. MTX = methotrexate 250 (mg/kg) s.c. at zero time; TdR = thymidine (250 mg/kg) s.c. 5.5 hr after MTX; and HxTdR = hypoxanthine (50 mg/kg) + thymidine (250 mg/kg) s.c. 5.5 hr after MTX. Cells were harvested at 6 hr and pulsed with [5-^{3}H]CdR (10 μCi/ml).

To determine whether the failures of TdR ± hypoxanthine protection in L1210/MP and L1210/FAMP cells were related to the greater suppressive effect of MTX on these cell lines, we attempted to produce an equivalent MTX effect in L1210/S by treating mice, bearing this tumor, with a higher dose of drug (MTX, 400 mg/kg, s.c.). This produced little change in the rate of incorporation by MTX-treated animals (28 per cent) compared with the smaller dose (31 per cent). Furthermore, TdR continued to offer a protective effect (50 per cent), whereas addition of hypoxanthine again restored incorporation with an overshoot to 146 per cent of control.

We pursued the hypothesis that failure of TdR protection in L1210/MP and L1210/FAMP might be related to increased sensitivity to MTX by considering only the data in the L1210/S line in which MTX suppressed incorporation to less than 30 per cent of control. In these four experiments, MTX inhibited incorporation to an average of 23 per cent of control. Despite the greater suppressive effect of MTX, however, TdR continued to offer substantial protection (71 per cent).

Thus, in cell lines with specific purine salvage defects, MTX produced a greater antimetabolic effect and TdR failed to influence this effect as seen for the parent line with intact purine salvage.

Survival studies. The results of survival experiments in L1210/S and L1210 mutant tumor-bearing mice are shown in Table 4. Treatment of mice with an LD_{100} of MTX and "rescue" with TIA prevented toxic deaths in animals bearing all tumor types. The extension of life span produced by the MTX–TIA combination was greater in the animals bearing the mutant cell lines than in L1210/S. Although different rescue schedules were used in some experiments, the results were consistent. Extension of life span for mutant lines treated with MTX and rescued with CF was also superior to that for L1210/S, although the differences were less marked. The median survival of the untreated groups of mice was comparable, indicating that the *in vivo* growth rates of the tumors were the same and arguing against collateral sensitivity on an immunologic basis. The nadirs of the cumulative daily weights of the treated groups confirm equivalent MTX effects on normal tissues and efficacy of TIA rescue.

DISCUSSION

MTX, by producing a folate-depleted state in DNA-synthesizing cells, interferes with the synthesis of thymidylate from deoxyuridylate and with *de novo* purine biosynthesis. The cytotoxic effects of MTX can be prevented *in vivo* and *in vitro* by supplying salvageable precursors in the form of thymidine and preformed purines [6, 11]. Hryniuk [7] demonstrated

Table 4. Thymidine–inosine–allopurinol (TIA) and citrovorum factor (CF) "rescue" from MTX effects in tumor-bearing mice*

	Median survival of untreated groups (days)	Extension in life span (days)		Nadir daily wt as % day 0 body wt
		TIA	CF	
L1210/S (N = 7)	8.8 ± 0.1	3.0 ± 0.2	2.4 ± 0.1	89.3 ± 0.9
L1210/MP (N = 7)	8.7 ± 0.2	5.7 ± 0.4	4.0 ± 0.3	90.4 ± 1.3
L1210/FAMP (N = 3)	10.3 ± 0.3	5.0 ± 0	4.3 ± 0.3	88.5 ± 3.5
L1210/555† (N = 2)	9.5‡	7	6	88.5

* Values are means ± S.E.M.

† Results in L1210/555 represent the means of two separate experiments with ten mice per experimental group per experiment.

‡ In the parallel studies with L1210/555 and L1210/S, median survival of untreated groups was identical.

that, in murine tumors, a purineless state may determine the cytotoxic effect of MTX. Therefore, the relative sensivity of the *de novo* purine biosynthetic pathway to folate depletion may be an important determinant of responsiveness to MTX. It has been shown that cells lacking the purine salvage enzyme HGPRT have accelerated *de novo* purine synthesis [12]. Thus, cells with impaired purine salvage may be more sensitive to MTX through either increased reliance on *de novo* purine synthesis or inability to use endogenous purines that may partially reverse the MTX effect. In man, thymidine, when administered alone, can prevent MTX toxicity [1, 2]. Since MTX inhibits *de novo* purine synthesis, the effectiveness of thymidine rescue presumably depends upon utilization of circulating purines. The importance of purine salvage has been demonstrated for a number of different organ systems in mammals [3, 13, 14] and for human bone marrow cells *in vitro* [15].

If purineless death is a mechanism for the antitumor effect of MTX in some human tumors, then variations in MTX sensitivity could be related to variabilities in purine salvage capacity. Furthermore, such a mechanism potentially offers greater antitumor selectivity of MTX with thymidine rescue.

In this study, we have produced murine tumor cell lines with impaired purine salvage to test the hypothesis that such impairment would result in enhanced sensitivity to MTX, especially when nucleoside "rescue" is employed. *In vivo* exposure to MTX produced greater suppression of DNA synthesis in cells with impaired purine salvage, despite *in vitro* tests suggesting identical MTX sensitivity with the parent cell line, L1210/S.

Furthermore, administration of thymidine did not reverse the MTX effect in mutant cells, although it produced a significant reversal in the parent line. *In vitro* studies of purine salvage indicate that, in L1210/MP, thymidine salvage was intact since, in combination with adenine, it provided significant protection against MTX (see Table 2 and Fig. 1). *In vivo*, therefore, the effect of MTX on L1210/MP and the failure of thymidine reversal may indicate a role for circulating purines in overcoming the effects of MTX.

It has long been known that resistance to a chemotherapeutic agent is often accompanied by collateral sensitivity to a second antitumor agent and this has been described for 6-MP resistant L1210 cells with respect to MTX [16]. Recent studies have emphasized the immunologic factors that may account for such observations [17–19]. However, where drugs are thought to produce collateral sensitivity on an immunologic basis, the tumors in question have demonstrated either a slower *in vivo* growth rate or a diminished capacity to cause death.

In the present studies, the mutant cell lines L1210/MP and L1210/555 were as effective as L1210/S in causing death, as indicated by the median survival times of untreated groups of animals. On the other hand, the longer median survival time of untreated animals bearing L1210/FAMP versus L1210/S suggests an antigenic change. If such an immunologic phenomenon contributed significantly to collateral sensitivity, however, the effect of MTX should have been maximal in L1210/FAMP, but it was not. Furthermore, cell line L1210/555, which was derived directly from L1210/FAMP, did not display evidence of antigenic change as manifested by prolonged median survival of untreated animals. These observations suggest that the observed collateral sensitivity to MTX was probably not on an immunologic basis and indicates a biochemical mechanism. Further studies are required to demonstrate the precise mechanisms of enhanced *in vivo* MTX sensitivity in these lines. Such mechanisms may also contribute to human tumor responsiveness to this drug.

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