Influence of Concomitant Infusion of Thymidine and Inosine on Methotrexate Activity in Normal and P388-bearing Mice*

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Abstract—*Combinations of thymidine and inosine (ranging from 0 to 7.5 mg/hr) were co-administered during a 72-hr continuous i.v. infusion of 3 μg/hr methotrexate in normal and P388 solid tumor-bearing DBA/2 mice. Methotrexate alone was lethal to all normal mice. Inosine at 1.0–7.5 mg/hr could reverse toxicity up to 100% while thymidine at 0.5–7.5 mg/hr was less effective (≤86% survival). Combinations of the nucleosides averted toxicity more effectively than either compound alone and in a synergistic manner. From P388 tumor-bearing mice 27% survived methotrexate and eventually died of tumor. Co-infusion of thymidine or inosine decreased the percentage of toxic deaths and caused an increase in life span of more than 50% as compared to untreated tumor-bearing mice. However, the diminishing effect on survival with thymidine and inosine doses above 0.5 mg/hr indicated loss of the antitumor effect of methotrexate. This was also observed with combinations of the nucleosides. The influence of thymidine on the antitumor effect of methotrexate was compared in L1210- and P388-bearing mice (both in ascites) with 13 μg/hr methotrexate for 48 hr and 4 mg/hr thymidine for 96 hr. The increase in life span for L1210-bearing mice (8.6 days, 96%) was significantly longer than that with P388-bearing mice (6.1 days, 56%), probably due to biochemical differences between these tumors. It is concluded that co-administration of inosine and/or thymidine allows the use of methotrexate doses otherwise not tolerated, though with loss of anti-tumor effect. The choice of the tumor model may greatly influence the outcome of in-vivo studies on the modulation of methotrexate action by nucleosides.*

INTRODUCTION

IN-VITRO studies have shown that thymidine (dThd) alone or in combination with a purine can reverse the cytotoxicity of methotrexate (MTX) to tumor cell lines [1–3] and mouse myeloid precursor cells [4]. In tumor-bearing mice, allopurinol, which increases purine availability, partly reversed the antitumor activity of MTX

against L1210 but not against P388 [5]. Tattersall et al. [6] showed an improved therapeutic index for MTX in L1210-bearing mice by co-administration of dThd. Although others confirmed this result with L1210-bearing mice, the reversal of MTX toxicity in normal mice was found to require both dThd and a purine [7, 8]. It is possible that, in tumor-bearing mice, killed tumor cells provide sufficient amounts of purines [8]. In continuous infusion experiments, Semon and Grindey [9] showed that either dThd or inosine (Ino) could reverse MTX-mediated host toxicity, but that Ino also blocked the antitumor effect.

The aims of the present study were (a) to further our understanding of the interactions of MTX

with dThd and Ino in normal and tumor-bearing mice using a continuous infusion system, and (b) to determine whether the choice of tumor model in the other studies [7–10] has influenced the results concerning the efficacy of MTX with co-administration of dThd plus purines.

MATERIALS AND METHODS

Chemicals

MTX was obtained from Pharmachemie BV (Haarlem, The Netherlands), while dThd and Ino were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Solutions of agents were prepared in 0.65% NaCl solution, adjusted to pH 7.0, and sterilized by passage through 0.22-μm Millipore filters. Powdered RPMI 1640 medium and fetal bovine serum were purchased from Grand Island Biological Co. Europe (Glasgow, U.K.). All other chemicals used were of analytical grade quality and obtained commercially.

Mice

Female DBA/2 mice (19–21 g) were obtained from IFFA/CREDO, Saint-Germain-sur-l'Arbreste, France. Mice were maintained on standard laboratory diet (RMH-B, Hope Farms BV, Woerden, The Netherlands) and water ad libitum.

Tumors

P388 leukemia cells, known to be sensitive to MTX [11], were kindly supplied by Dr G. Atassi from the Institute Jules Bordet, Brussels, and were maintained in suspension culture as described elsewhere [12]. Cultures were diluted with fresh medium to a density of 10^5 cells/ml twice weekly and 24 hr prior to inoculation into mice. In all experiments, 10^6 P388 cells in 0.5 ml 0.9% NaCl were inoculated s.c. in the cervical region of recipient mice, and drug infusions were initiated 24 hr later. Maintenance of L1210 cells was identical to that for P388. In those experiments in which L1210 and P388 were compared, cells were inoculated i.p. to allow further comparison with studies reported by others [9, 10]. In each case, survival was calculated from the day of tumor inoculation, termed day 0.

Infusions

Drugs were administered by continuous i.v. infusion to unrestrained mice by a technique as essentially described by others [9, 10, 13]. Drugs were infused at a rate of 0.5 ml/hr for 72 hr, unless otherwise indicated. Mice still living on day 30 were scored as survivors.

Analysis of plasma nucleoside levels

Nucleoside concentrations in plasma samples collected before and during infusion were determined by high-pressure liquid chromatographic methods as described elsewhere [14].

RESULTS

Reversal of MTX toxicity by nucleosides in normal mice

Continuous i.v. infusion of MTX at a rate of 3 μg/hr for 72 hr resulted in a 100% kill of normal mice ($n = 14$). Co-infusion of nucleosides provided dose-dependent protection against MTX toxicity (Fig. 1). Analysis of the data with Fisher's exact probability test [15] indicated that significant ($P < 0.05$) protection from MTX toxicity was provided by dThd at doses of 1, 3 and 7.5 mg/hr. At these doses the dThd effect was maximal, resulting in 83% survival. Ino caused complete reversal of MTX toxicity at a dose of 7.5 mg/hr ($P < 0.05$). Lower doses (1.0 and 3.0 mg/hr) provided only partial protection against MTX toxicity (Fig. 1). In mice treated with 10 μg/hr MTX, dThd had no effect on MTX toxicity, while Ino at 7.5 mg/hr produced full reversal (data not shown; ref. [16]).
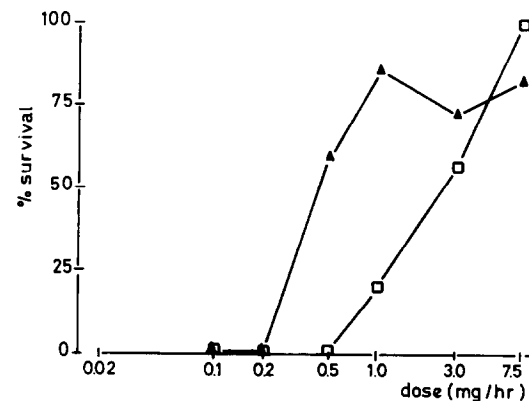

Fig. 1. The relationship between survival and dose of dThd ($\blacktriangle$) or ($\square$) Ino used for the reversal of MTX (3 μg/hr) toxicity in healthy DBA/2 mice. Each point represents at least 5 mice.

If the effects of dThd and Ino on MTX toxicity were additive, the expected fraction of mice killed by MTX plus a dThd–Ino combination would be the product of the fraction killed by MTX plus Ino and the fraction killed by MTX plus dThd. Co-infusion of a dThd–Ino combination was more effective in preventing mortality from MTX than either nucleoside alone. Moreover, the survival observed after co-infusion of the dThd–Ino combination was higher than expected on the basis of a merely additive reversal effect (Table 1). Excluding the combinations of 0.2 mg/hr dThd with 0.5 or 1.0 mg/hr Ino, all nucleoside combinations tested resulted in 100% protection against lethal MTX toxicity ($P < 0.05$; Fisher's exact test [15]).

Table 1. Synergism between dThd and Ino in reversal of MTX toxicity

dThd dose (mg/hr)	Ino dose (mg/hr)	Percent survival of mice treated with MTX and nucleosides*	
		Calculated†	Measured‡
0.2	0.5	0	50
	1.0	20	43
	3.0	57	100
	7.5	100	100
1.0	0.5	86	100
	1.0	89	100
	3.0	94	100
	7.5	100	100
7.5	0.5	83	100
	1.0	86	100
	3.0	93	100
	7.5	100	100

*Mice were given 3-day infusion with 3 μg/hr MTX and dThd and Ino as indicated.

†Survival was calculated from the product of the percentage kill after rescue with dThd alone and the percentage kill after rescue with Ino alone (i.e. value expected when reversal is additive).

‡The data represent the surviving fraction of groups of at least 5 mice.

Effect of nucleoside infusions on MTX antitumor action against P388 leukemia

Subcutaneous inoculation of 10^6 P388 leukemia cells resulted in the growth of a large solid tumor with extensive metastatic disease in lungs, liver and spleen, leading to death within 15 days. In contrast with normal mice, not all of the tumor-bearing animals were killed by a 3-day infusion of MTX at 3 μg/hr, 27% of the animals surviving toxicity and eventually dying of tumor. During the first 6–7 days following MTX infusion, drug toxicity in these animals was apparent from bristling hairs, high back-bones and a 25% weight loss. This was followed by a gradual increase in weight and improved condition of the mice until the development of large subcutaneous tumors.

Co-administration of dThd or Ino with MTX provided protection against drug toxicity. The increase in lifespan (ILS) was highest with the lowest nucleoside doses used (Fig. 2, Table 2). All animals surviving beyond day 5 eventually died of tumor. The ILS obtained with MTX plus ≤1.0 mg/hr nucleoside was significantly higher than the one obtained with MTX plus 3.0 and 7.5 mg/hr nucleoside ($P < 0.05$, Wilcoxon's test [15]).

Combinations of dThd and Ino were also tested for reversal of MTX toxicity in P388-bearing mice. Table 2 shows the relationship between the nucleoside doses used, the median survival time and the percentage of mice protected from MTX toxicity. As observed with co-infusion of single
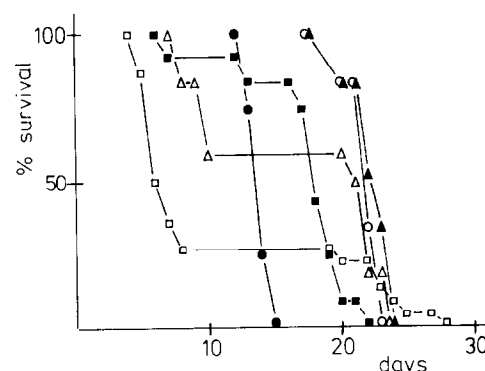

Fig. 2. Survival curves of P388-bearing mice treated with 0.65% NaCl (●), with MTX (3 μg/hr) plus no dThd (□), MTX plus 0.2 mg/hr dThd (▲), MTX plus 0.5 mg/hr dThd (△), MTX plus 1.0 mg/hr dThd (○) or MTX plus 7.5 mg/hr dThd (■). Each curve represents at least 6 mice.

nucleoside, combinations of the nucleosides resulted in reduced median survival with increasing nucleoside doses.

Comparison between P388 and L1210 in response to MTX plus dThd

To allow comparison with data on L1210 reported by others [8, 10], a parallel experiment was performed with one group of P388-bearing and one group of L1210-bearing DBA/2 mice. In this experiment, 10^6 tumor cells were inoculated i.p. Untreated L1210-bearing mice had a median survival of 9 days (range 8–10 days), whereas the median survival of untreated P388-bearing mice was 11 days (range 9–12 days). Both groups were treated simultaneously with MTX at 13 μg/hr (16 mg/kg/day) and with dThd at 4 mg/hr (5 g/kg/day) for 48 hr, followed by 4 mg/hr dThd for another 48 hr. This was the optimal regimen reported by Semon and Grindey [9] which yielded a 125% ILS over controls in L1210-bearing DBA/2 mice. In our hands, this schedule afforded a 96% ILS in L1210-bearing mice but only a 56% ILS in P388-bearing animals. This difference was significant with a $P < 0.05$ (generalized t test [15]).

Plasma base and nucleoside levels

Normal DBA/2 mice had dThd plasma levels of 2.8 ± 2.4 μM (mean ± S.D.). Infusion of 1 and 3 mg/hr dThd yielded mean plasma concentrations of 10 and 25 μM respectively. Infusion of 7.5 mg/hr resulted in dThd plasma levels of over 200 μM (Fig. 3). Baseline levels for adenine, hypoxanthine and Ino were below the limit of detection (<0.5 μM).

DISCUSSION

Constant infusions of MTX to DBA/2 mice at a rate of 3 μg/hr for 72 hr yield plasma drug concentrations of 0.1 μM [17]. In the present study

Table 2. Reversal of toxicity of 3 μg/hr MTX by co-infusion of combinations of dThd and Ino and effect on antitumor activity of MTX of this co-infusion

Addition* (mg/hr)					Increase in
Ino	dThd	n†	% tumor death‡	Median survival§ (days)	median survival‖ (%)
0	0.0	22	27	6.0	-57
	0.2	6	100	22.5	61
	0.5	6	67	21.5	54
	1.0	6	100	22.0	57
	7.5	12	92	18.0	29
0.5	0.0	6	67	21.5	54
	0.2	6	50	15.0	7
	1.0	6	83	20.0	43
	7.5	6	100	18.5	32
1.0	0.0	6	100	21.0	50
	0.2	6	83	22.0	57
	1.0	5	80	20.0	43
	7.5	6	100	19.0	36
3.0	0.0	3	100	19.0	36
	0.2	6	100	19.5	39
	1.0	5	80	18.0	29
	7.5	4	75	16.0	14
7.5	0.0	4	100	18.5	32
	0.2	6	100	16.5	18
	1.0	5	100	17.0	21

*All mice were infused for 72 hr with 3 μg/hr MTX plus additions as indicated.
†No. of animals per experiment.
‡The percentage of mice dying of tumor after having survived MTX toxicity.
§Life span from the day that the mice received P388 cells s.c.
‖Increase in life span as compared to untreated controls (median survival: 14 days; range: 13–15 days).
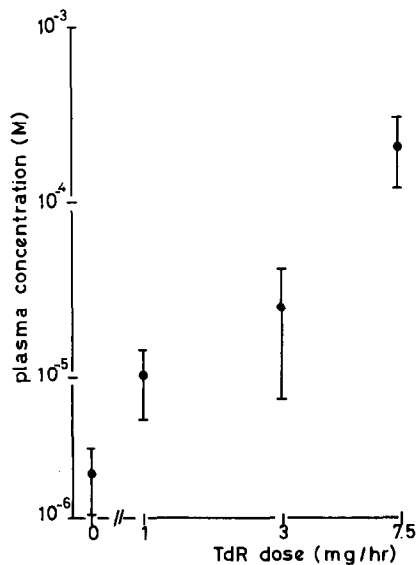

Fig. 3. Plasma dThd concentrations during long-term i.v. infusions in normal DBA/2 mice. The vertical bars indicate the S.E.M.

this appears to be lethal to all animals treated. Co-infusion of either dThd or Ino provided substantial protection of mice from MTX toxicity. dThd doses of 1–7.5 mg/hr provided a maximal protection of up to 83% of the MTX-treated mice. Ino at 7.5 mg/hr afforded complete reversal of MTX lethality (Fig. 1, [16]). The limited protective effect of dThd against MTX toxicity in mice as opposed to the complete reversal by Ino was also observed by others [6, 9]. The rapid clearance of dThd [16] compared with MTX could account for the lack of complete rescue of mice from MTX toxicity, since in these experiments dThd infusion was not extended beyond the end of MTX infusion.

Another explanation of this difference in protective effect could be our finding that in normal DBA/2 mice circulating dThd levels are higher than purine levels. However, others have found relatively high plasma purine concentrations [9]. Differences in handling of blood samples could account for discrepancies in the determination of plasma purine content in view of the rapid turnover of purine nucleotides in erythrocytes [18]. Catabolism of intracellular

adenine nucleotides at millimolar concentrations followed by leakage of catabolites could lead to substantial increase in plasma purine concentrations [19].

Ino at doses as low as 0.5 mg/hr enhanced the rescue effect of dThd. Interestingly, combinations of Ino at 0.5–3 mg/hr with dThd at 0.2 mg/hr clearly showed synergistic reversal of MTX toxicity (Table 1, ref. [16]). Others did note that dThd plus Ino protected normal [8, 9] and tumor-bearing [7] mice better than either compound alone. However, no conclusive evidence of synergism was provided in these studies. The results of a clinical study of MTX combined with dThd, Ino and allopurinol also suggested that the addition of purine to dThd improves rescue [20].

A recent in-vitro study from our laboratory showing that purines can stimulate the conversion of dThd to dTTP [21] offers a possible explanation for this synergism. Purines, when given in combination with dThd, would not only alleviate the MTX-induced purineless state but also facilitate abolishment of the thymidylate depletion by providing ATP for kinase activities for the formation of dThd nucleotides. This is in agreement with work by others who have demonstrated that MTX can hamper ATP requiring enzyme reactions [22].

Tumor-bearing mice were less sensitive to MTX and required lower doses of nucleosides for complete protection against MTX (Tables 1 and 2). This may be due to an increased availability of circulating salvage metabolites released through lysis of MTX-sensitive tumor cells, as has been suggested previously [8, 23].

Mice treated with MTX alone that survived MTX toxicity had an ILS of 69% (Fig. 2). Co-administration of MTX and nucleosides reduced antitumor effect as well as toxicity of MTX. Treatment with MTX plus nucleosides consistently led to an ILS lower than the 69% obtained with MTX alone (Table 2). There appeared to be an inverse relationship between the ILS observed and the dose of nucleosides used for reversal of toxicity. No conclusions could be drawn regarding synergistic effects on suppression of antitumor effect.

L1210 cells are known to be less sensitive to MTX than P388 cells [11]. Using an MTX–dThd regimen providing optimal antitumor effect with protection against MTX toxicity [9, 10], we found a significantly greater ILS with L1210-bearing mice than with P388-bearing mice. This may be explained by the observation made by Kinahan *et al.* [24] that L1210 cells *in vitro* do not accumulate sufficient dTTP levels in the presence of MTX plus dThd, in contrast to other cells. In addition, we have demonstrated earlier that MTX toxicity to L1210 cells *in vitro* is not reversed by low dThd plus high Ino [12]. Yet the present in-vivo study shows that this nucleoside combination renders MTX inactive against P388 cells. These data suggest that the improved therapeutic effects of MTX plus dThd reported by others [7–10] may be attributed to the differential response of L1210 cells to dThd in the presence of MTX.

Our results with concomitant infusion of MTX and nucleosides in P288-bearing mice are similar to the results from clinical studies with MTX and dThd [25–29] in which the combination was found to have limited antitumor activity, despite the use of minimal doses of dThd which produced only modest increase in plasma dThd concentrations [27–29].

Not only did we confirm that both dThd and Ino are effective agents for reversal of MTX toxicity, but we also demonstrated that these nucleosides in combination act synergistically. However, the inverse relationship between the antitumor effects of MTX against P388 leukemia cells and the nucleoside doses used precludes substantial improvement of the therapeutic index. The promising results of earlier experimental studies on co-administration of MTX plus nucleosides may have been limited to L1210 leukemic cells.

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