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Significance of Variation in Serum Thymidine Concentration for the Marrow Toxicity of Methotrexate

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Summary. *Thymidine (dThd) concentrations have been measured in the sera of normal subjects and solid tumor cancer patients by means of a sensitive high-pressure liquid chromatographic assay to determine whether natural and methotrexate (MTX)-induced fluctuations were large enough to alter the toxicity of MTX to marrow. The mean concentration in normal subjects with measurable levels was 1.3×10^{-7} M (range $< 4 \times 10^{-8}$ to 6×10^{-7} M). In cancer patients it was 2.0×10^{-7} M (range $< 4 \times 10^{-8}$ to 8.7×10^{-7}), and in malignant effusions 1.2×10^{-7} M (range $< 4 \times 10^{-8}$ to 2.2×10^{-7} M). The wide range of variation in random samples was also found when multiple samples were obtained from the same patient during a 24-h period where dThd concentration varied from a minimum of two- to greater than six-fold. Treatment with MTX 3 mg/m² caused an average 59% reduction in serum dThd during the first 24 h after injection during nine courses of therapy. dThd was tested for its ability to modulate the toxicity of MTX to human granulocate colony-forming units in culture across the concentration range found in vivo: changes in dThd concentration equivalent to normal fluctuations in vivo altered colony survival by 31% to > 72%. A reduction in culture dThd equivalent to that produced in vivo by high-dose TMX increased colony kill by 25%. The results indicate that in vivo variations in serum dThd are in an appropriate range and of a sufficient magnitude to alter the toxicity of MTX to marrow, and they demonstrate that MTX can modulate its own toxicity by reducing serum dThd.*

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The abbreviations used are: CFU-GM, granulocyte/macrophage colony-forming units; dThd, thymidine; 5-FU, 5-fluorouracil; FdUrd, fluorodeoxyuridine; HPLC, high-pressure liquid chromatography; MTX, methotrexate

Introduction

dThd is readily transported from the extracellular environment, phosphorylated via salvage pathway enzymes, and incorporated into the DNA of proliferating cells. When present in a sufficient concentration, dThd can block the toxicity of drugs whose activity depends on reduction of intracellular thymidylate pools, such as trimethoprim and the sulfonamide antifols [28], MTX [9], and FdUrd [12]. Recently, dThd was found to protect against the toxicity of high doses of MTX in both mice [25, 29] and man [6, 14, 15]. dThd can also modulate the cellular pharmacology of several important antimetabolites: it increases the incorporation of 5FU into RNA [3, 24], improves its therapeutic ratio against murine tumors [21], and markedly augments the toxicity of 5FU [31, 32] in patients. It also interacts synergistically with ara-C [2, 8, 11]. However, little information is available on whether normal fluctuations in the endogenous dThd concentration are in an appropriate range, and of a sufficient magnitude, to modulate the toxicity of antimetabolites in vivo.

In studies of mouse marrow, dThd provided increasing degrees of protection for CFU-GM against MTX toxicitiy in culture over the concentration range of 10^{-7} to 10^{-5} *M* [22]. A steeper dose-response effect was suggested for human marrow by the results of a recent clinical trial directed at determining the minimum dose required to rescue patients from high-dose MTX exposures [17]. To ascertain whether variations in the endogenous circulating serum dThd concentration could contribute to the variable toxicity of MTX [10], we measured the dThd concentration in biological fluids of representative groups of normal subjects, solid tumor patients, and patients receiving high-dose MTX chemotherapy. The range of dThd concentrations found in vivo was compared

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with that required to modulate the toxicity of MTX to human marrow CFU-GM in culture.

Materials and Methods

Subjects and Samples. Blood samples were obtained at random times from 20 normal subjects and eight patients with advanced solid tumors (non-oat cell, lung 3; soft tissue sarcoma 2; head and neck 1; ovary 1; colon 1). Seven normal subjects were sampled every 2–4 h for 1 day, during which time no restrictions were placed on diet or activity. Blood samples were also obtained from five patients with solid tumors (non-oat lung 2; soft tissue sarcoma 2; head and neck 1) who were receiving high-dose MTX chemotherapy as part of a phase 1 trial [17]. In this group of patients blood was drawn just before, and 24 h after, rapid injection of MTX 3.0 g/m^2. Blood was obtained by venipuncture, and was either allowed to clot or anticoagulated with 20 U heparin/ml. Serum and plasma were separated by centrifugation at 4° C within 4 h and stored at −30° C until analyzed. Ascitic and pleural fluid samples were obtained by thoracentesis and paracentesis from nine patients with malignant effusions (non-oat cell lung 5; ovary 3; breast 1).

HPLC Assay. Serum samples were prepared for analysis by precipitation of protein with 0.1 volume 4.4 N perchloric acid, followed by clarification by centrifugation, and neutralization with Alamine/Freon TF as described by Khym [19]. Neutralized aqueous material (100 µl) was injected on each run. A Waters HPLC system (Waters Associates, Milford, Mass.) with a reverse-phase Bondapak C18 column was used for all determinations. Column output was monitored at 254 and 280 nm, and peak heights and areas were electronically integrated. The column was eluted with a 15-min linear gradient starting with a 5-m*M* potassium phosphate buffer at pH 3.34 and ending with 15% 60/40 v/v acetonitrile/water at a flow rate of 1.5 ml/min at room temperature. dThd standards were prepared in water with dThd obtained from the National Cancer Institute. Concentrations of pure standards were checked by optical density, and dThd peaks were identified by retention time, absorbance ratios at 254 and 280 nm, and co-chromatography of standards [20].

Plasma Protein Binding. The fraction of dThd bound to plasma protein was determined by adding trace amounts of (^{3}H)-dThd and various concentrations of cold dThd to normal human plasma anticoagulated with heparin 20 U/ml at room temperature. Separation of free and bound dThd was accomplished with Amicon CF 50 A filter cones (Amicon Corp., Lexington, Mass. USA), which retain protein at > 50,000 mol. weight. The concentration of (^{3}H)-dThd in plasma water was determined by scintillation counting.

Assay of Granulocyte/Macrophage Colony-forming Units. Marrow samples from patients without hematologic disease were cultured in the continuous presence of 10^{-5} *M* MTX and 10^{-4} *M* hypoxanthine in 0.8% methyl cellulose with modified McCoy's 5A medium containing 15% calf serum as described elsewhere [16]. Medium conditioned by pokeweed mitogen-stimulated lymphocytes was added at a final concentration of 10% as a source of colony-stimulating factor. Triplicate cultures were incubated at 37° C in 7.5% CO_2, and aggregates of greater than 40 cells were scored as colonies on days 7–10. The hypoxanthine and dThd concentrations of fetal calf serum used in the medium was determined by HPLC: the concentration of dThd in the medium contributed by the calf serum was $< 1.5 \times 10^{-8}$ *M*.

Results

Figure 1 shows that the height of the dThd peak resolved by HPLC was linearly related to the dThd concentration when standard amounts of dThd were added to dialyzed serum. By gradient elution, the thymidine peak was consistently completely resolved from nearby peaks, and the lower limit of sensitivity of the assay was 4×10^{-8} *M*. The coefficient of variation for five repeated measurements of serum containing the same concentration of thymidine is listed in Table 1, and varied from 14%–16% over the dThd concentration range of 4×10^{-8} *M* to 10^{-6} *M*. Table 1 also lists the percent recovery of dThd and the percentage that was freely filterable over the same concentration range. The recovery of known amounts of dThd added to human serum varied from 96%–106%. These high recovery rates are consistent with the results of ultrafiltration experiments that demonstrated that 96%–99% of the dThd was free in plasma water rather than bound to plasma proteins. Also in accord with these results was the finding the < 1% of added (^{3}H)-dThd was precipitated from human plasma by perchloric acid.

Figure 2 compares the dThd concentrations found in random samples of serum obtained from normal subjects, in serum from patients with solid tumors,

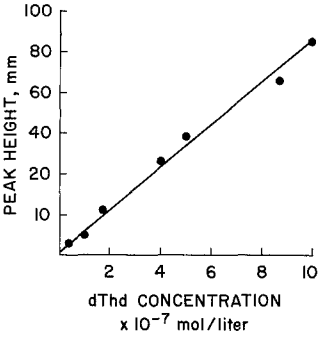

Fig. 1. Linear relationship between dThd concentration and the height of dThd peak in chromatograms of human serum. The lower limit of sensitivity was 4×10^{-8} *M*. The slope of the line was determined by least mean squares fitting

Table 1. Characteristics of HPLC assay of serum thymidine

dThd Concentration (Molar)	Coefficient of variation[a]	Percent recovery by HPLC (± SD)	Percent protein-bound (± SD)
4×10^{-8}	0.14	106 ± 14	96.1 ± 2.2
10^{-7}	0.10	92 ± 9	98.4 ± 1.0
10^{-6}	0.16	96 ± 16	98.6 ± 4.2

[a] Five repeated assays

and in malignant pleural and peritoneal effusions. The geometric mean concentration for the 17 of 20 samples from normal subjects with detectable levels was $1.3 \times 10^{-7} M$; the concentration range was $< 4 \times 10^{-8} M$ to $6.0 \times 10^{-7} M$. The mean concentration in the 11 of 12 serum samples from cancer patients with measurable levels was $2.0 \times 10^{-7} M$, and the levels ranged from $< 4 \times 10^{-8} M$ to $8.7 \times 10^{-7} M$. Among nine malignant effusions studied, only three had measurable concentrations of dThd, with a mean of $1.2 \times 10^{-7} M$; they ranged from $< 4 \times 10^{-8} M$ to $2.2 \times 10^{-7} M$. These mean levels must be considered as estimates only, because in each group the dThd concentration in some samples was below the limit of sensitivity of the assay. The mean concentration in normal subjects, solid tumor cancer patients, and malignant effusions did not differ significantly from each other. The large fraction (6/9) of malignant effusion samples with dThd levels below the limit of sensitivity of the assay suggests that the average dThd concentration in these fluids may be significantly lower than that in serum.

The most important finding to emerge from this set of measurements was that the range of dThd concentrations in all three groups of samples was very wide. The maximum value exceeded the minimum by 15-fold in the normal subjects, 22-fold in the cancer patients, and 5-fold in the effusions, indicating that in man dThd is not regulated within narrow bounds. This very wide variation in serum dThd was confirmed when concentrations were determined in serum samples obtained every 2–4 h for a 24-h period in seven normal subjects (Fig. 3). The maximum dThd concentration was at least two fold greater than the minimum in every subject, and in three subjects there was a greater than five fold change in dThd concentration during a single day. The variance in the dThd concentrations significantly exceeded that which could be accounted for by the variance inherent in the HPLC assay ($P < 0.01$, F-test). There appeared to be no consistent pattern of fluctuation in dThd level during the day, and variations could not be related to meals, activity, or sleep. To further assess the contribution of dietary dThd to the variance in serum levels, one patient was given dThd 1 g/m^2 IV and then, when the serum dThd concentration had returned to baseline, the same dose was given PO. Figure 4 shows that whereas administration of dThd by the IV route caused a marked increase in serum dThd, administration PO caused no change. This indicates that even when administered in large doses, the gastrointestinal absorption of dThd, or its first metabolite thymine, does not contribute significantly to serum dThd.

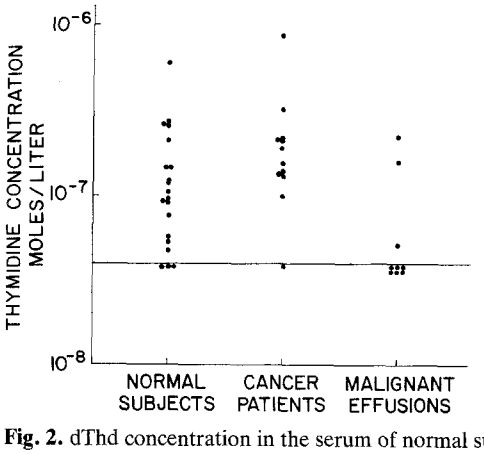

Fig. 2. dThd concentration in the serum of normal subjects, in the serum of cancer patients with solid tumor, and in malignant effusions measured by HPLC, as described in *Materials and Methods*. Samples were drawn at random times of day. *Horizontal line* indicates lower limit of assay sensitivity; *horizontal bar* indicates geometric mean of samples containing measurable concentrations of dThd

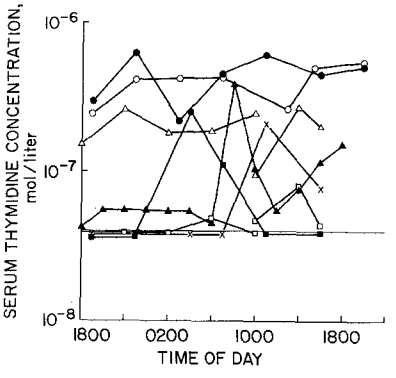

Fig. 3. Variation in serum dThd concentration in seven normal subjects sampled every 2–4 h for a 24-h period. *Horizontal line* indicates lower limit of sensitivity of HPLC assay

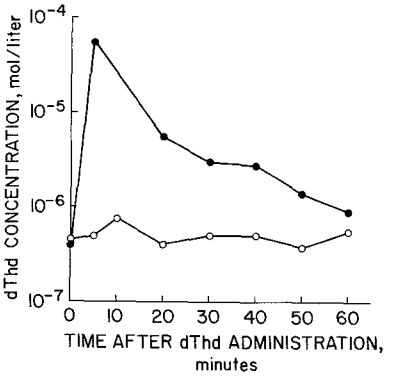

Fig. 4. Serum concentrations of dThd after IV (●——●) and PO (○——○) administration of dThd 1 g/m^2. Time zero samples were obtained before dThd administration

Since MTX exerts some of its cytotoxic effect by depleting intracellular pools of thymidylate, it was of interest to determine the effect of MTX on total body dThd as reflected by serum dThd concentration. Figure 5 shows the change in serum dThd during nine courses of treatment in five patients receiving high-dose MTX therapy. Serum samples were obtained just before, and 24 h after, rapid IV administration of MTX 3.0 g/m^2. dThd concentration fell during all nine courses, with decreases averaging 59% and ranging from 39%–83% ($P < 0.01$, one-tailed t-test) (Fig. 5).

The significance of the in vivo changes in serum dThd for the toxicity of MTX to normal marrow was examined by exposing human CFU-GM in vitro to 10^{-5} M MTX and variable concentrations of dThd in the presence of excess hypoxanthine. Fig. 6 shows that across the concentration range of 4×10^{-8} to 10^{-6} M dThd, the dose-response curve for protection against MTX cytotoxicity was steep, and that the mean serum level of 1.3×10^{-7} M in normal subjects lies on the middle portion of the curve, so that relatively small changes in the dThd concentration in the cultures above and below the mean resulted in large differences in colony survival. For example, the dThd concentration varied from a minimum of two- to more than sixfold in a single 24-h period in vivo in normal subjects. Such changes in the dThd concentration in the CFU-GM cultures resulted in 31% to greater than 72% change in colony survival. In vivo, high doses of MTX caused on average a 59% decrease in serum dThd from a mean pretreatment level of 2.1×10^{-7} M. Such a change in the dThd concentration in the CFU-GM cultures increased the toxicity of MTX by a factor of 25%.

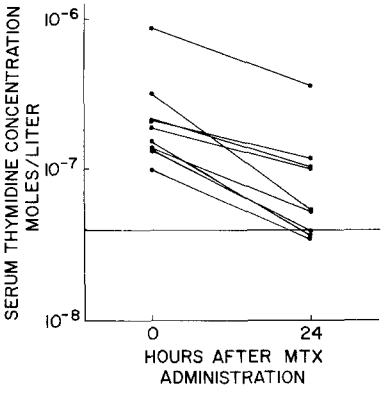

Fig. 5. Change in serum dThd concentration induced by MTX. Serum samples were obtained just before, and 24 h after IV injection of 3 g MTX/m^2. *Horizontal line* indicates lower limit of sensitivity of HPLC assay; *horizontal bar* indicates geometric mean

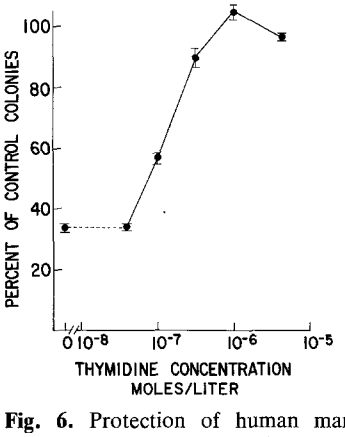

Fig. 6. Protection of human marrow CFU-GM against the cytotoxicity of MTX by dThd, measured by colony survival in vitro. Cells were cultured in methyl cellulose with medium containing MTX 10^{-5} M, hypoxanthine 10^{-4} M, and graded concentrations of dThd (zero indicates no dThd added). Each *point* represents the mean of triplicate plates in three experiments; *vertical bars* indicate standard error

Discussion

The most important finding to emerge from this study was that the variation of dThd serum concentration in normal subjects and cancer patients was of a magnitude sufficient to significantly modulate the toxicity of MTX to human CFU-GM when marrow was exposed to MTX in vitro, and that in large doses MTX decreased serum dThd level enough to increase its own toxicity. The clinical toxicity of MTX is notoriously variable [10]. Differences from patient to patient and course to course in the pharmacokinetics of MTX itself account for some of this variability. However, the results of this sutdy suggest two additional explanations: first, differences in the endogenous concentration of dThd present in the serum at the start of MTX exposure; second, differences in the degree to which MTX lowers the endogenous dThd concentration further. The magnitude of these fluctuations and effects is in the same range as is relevant for MTX cytotoxicity to human marrow. We found that for human marrow CFU-GM exposed to MTX in vitro, small changes in dThd concentration produced quite large differences in colony survival in the dThd concentration range 4×10^{-8} M to 10^{-6} M. Although any extrapolation of in vitro results to the in vivo situation must be made with caution, in this case the importance of small changes in serum thymidine concentration has been confirmed by the results of a recent clinical study of dThd rescue, in which a similar steep dose-response

effect for dThd was identified in vivo [17]. A dThd dose of 1.0 g/m^2, which produced less than a two fold increase in serum dThd concentration, was sufficient to prevent significant toxicity after an otherwise lethal dose of 3.0 g MTX/m^2 in most patients. Two additional clinical studies of MTX and dThd support the importance of this concentration range [6, 14]. Both concluded that elevation of serum dThd to the range of 10^{-6} M resulted in almost complete protection [6] or rescue [14] of patients from the toxic effects of even very long-term exposures to high doses of MTX. The dThd dose-response in this concentration range is also consistent with a reported K_m for the transport and phosphorylation of dThd in the range of 5×10^{-7} M in various mammalian cells [9, 23] and with the finding that dTTP pool size [1, 18] and rate of uptake of dThd into acid-soluble and -insoluble pools [4, 5] in various cell lines rise rapidly with increases in the extracellular concentration of dThd in the order of 10^{-7} M.

The close correspondence between the clinical trials and the studies of CFU-GM in culture with regard to critical concentrations of dThd also suggest that the serum dThd concentration is probably an accurate reflection of the concentration of dThd in the microenvironment of the marrow cells in vivo. However, further information both on the dThd concentration in the marrow microenvironment and on the ability of conventional doses of MTX to modulate serum dThd concentration is needed before the contribution of either fluctuations in endogenous dThd levels or modulation of these levels by MTX to the variable toxicity of MTX can be fully evaluated.

The wide variation in serum dThd concentration, both from person to person and during a 24-h period in the same subject, indicates that the dThd is not a closely defended constituent of blood. Marked variation in the serum dThd concentration in randomly drawn samples has been reported previously by investigators using radioimmunoassays for the measurement of dThd [6, 13, 14]. The mean endogenous dThd concentrations reported from these studies were 2.0×10^{-7} M and 1.6×10^{-7} M [14] for patients with solid tumors and 3.6×10^{-7} M for normal subjects [13], and these values agree closely with those determined by HPLC in this study. Holden et al. [13] reported that the mean serum dThd concentration was significantly higher in patients with leukemias and lymphomas than in normal subjects. In contrast, with a relatively small number of samples, we did not find a higher serum dThd concentration in patients with a variety of nonlymphomatous solid tumors. This was not unexpected, since the death rate of cells in most slow-growing solid tumors is very low relative to the 10-min half-life of thymidine in man [6].

In this study we were not able to identify the source of the marked variation in serum dThd concentration. The only three possible sources of serum dThd are dietary thymine or dThd, de novo synthesis from aspartate and carbamyl phosphate, and breakdown of senescent cells. Dietary thymine and dThd are not likely to constitute important sources of serum dThd. Thymidine is rapidly degraded in the gut and oral administration of 1 g pure dThd/m^2 did not change serum dThd at all. Although thymine is absorbed to some extent, gram quantities are required to reverse the thymidylate deficiency in pernicious anemia [26]. In addition, thymine is preferentially catabolized rather than converted to dThd [7]. In the doses used in this study, MTX causes destruction of large numbers of normal cells in the gastrointestinal epithelia and marrow, yet the net effect of MTX therapy was a reduction in serum dThd. This suggests that breakdown of cells was a substantially less important contributor to serum dThd than de novo synthesis, and thus implicates changes in de novo synthesis as the major source of variability of serum dThd. Several types of cells, including macrophages and fibroblasts [27, 30], secrete dThd into their environment in vitro, but at present there is no information about which tissues contribute to serum dThd in vivo.

This study provides the first example in man of an antimetabolite modulating its own toxicity by influencing the serum concentration of a salvage pathway metabolite. In the case of MTX in large doses, this effect is clearly of sufficient magnitude in some patients to have an impact on clinical toxicity, and possibly also on antitumor activity and selectivity. This concept, that the ability of a drug to control local and systemic dThd concentrations may be an important determinant of its activity, has particular relevance for the use of the fluorinated pyrimidines, which also in part exert their cytotoxic effect through reduction of intracellular thymidylate pools. The importance of serum dThd concentration for MTX toxicity in vivo also identifies control of salvage pathway metabolite concentration as a fulcrum with which to further improve the teherapeutic ratio of antimetabolites.

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