Thymidine and Hypoxanthine Requirements of Normal and Malignant Human Cells for Protection against Methotrexate Cytotoxicity

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ABSTRACT

The thymidine (dThd) and hypoxanthine (Hyp) requirements for protection of normal human granulocyte-macrophage colony-forming units, B-, and T-cells against methotrexate-induced inhibition of proliferation were compared to requirements of three malignant solid-tumor cell lines recently established from human tumor xenografts and of the CCRF-CEM T-cell leukemia. In the presence of excess Hyp, the concentration of dThd that provided 50% protection (PC50) was 0.2 μM for granulocyte-macrophage colony-forming units and normal T-cells; the dThd PC50 for malignant cells was five- to seven-fold higher, while that for B-cells was 10- to 40-fold higher. An accurate Hyp PC50 for granulocyte-macrophage colony-forming units could not be established; however, the Hyp PC50 for T-cells was 0.8 μM, while that for malignant cells was four to 30 times greater and that for B-cells, two to six times greater. All cells tested were dependent on both salvage pathway metabolites for protection. Physiological ranges for dThd and Hyp in humans were established using newly developed high-pressure liquid chromatographic techniques to measure concentrations in normal subjects and solid-tumor cancer patients. In normal subjects, the geometric mean serum dThd was 0.13 μM (range, 0.04 to 0.6 μM), and the mean plasma Hyp, 0.51 μM (range, 0.2 to 1.9 μM); cancer patients did not differ significantly. Neither excess dThd nor excess Hyp provided significant protection for any cell in the presence of average physiological concentrations of the second metabolite. The Hyp concentration in 14 freshly aspirated marrow specimens averaged 11 μM which was high enough to make protection of marrow dependent only on dThd. Relative to the wide range of physiological dThd and Hyp concentrations, the differences in PC50 were not large enough to suggest that administration of protective doses of dThd or Hyp would increase the therapeutic ratio. These results provide an explanation for why, in vivo, dThd alone is sufficient to protect patients against myelosuppression.

INTRODUCTION

MTX interferes with cell proliferation by depleting intracellular reduced folates that are required for the de novo synthesis of thymidylate and inosinate (39, 40). The degree to which inhibition of proliferation is due to reduction of thymidylate synthesis as opposed to purine synthesis varies widely between different cell types (11, 31, 46, 48, 50, 51). In a few types of cells, the action of MTX has been attributed mainly to induction of a thymineless state (5, 6, 43, 50), whereas, in many others, cytotoxicity has been attributed primarily to a purineless state (4, 15, 25, 27). The relative effect of MTX on thymidylate and de novo purine synthesis is also a function of MTX concentration and duration of exposure (6, 14, 26, 33, 43, 46, 58). Several animal trials (45, 49) suggest that the therapeutic ratio of MTX may be improved by combining MTX with either dThd or inosine. The biochemical basis for modulation of MTX selectivity by these compounds is not fully understood (40). However, it is clear that if there are large differences between normal and malignant tissues with respect to the antipurine versus antithymidylate effect of MTX or with respect to their ability to utilize dThd or purines via the salvage pathways (8, 36), then there is substantial potential for improvement in the therapeutic ratio by combining MTX with either dThd or purine. It is also apparent that variations in plasma dThd or purine concentrations or locally high concentrations of either of these salvage pathway metabolites in the interstitial fluid of the tumor, gut, or marrow may influence the selectivity of MTX. Techniques with enough sensitivity to measure plasma dThd and Hyp have only recently become available (18, 28, 57). To assess potential clinical applications of MTX in combination with dThd or purines, we have examined the dThd and Hyp requirements of normal human marrow CFU-GM, T-cells, and Epstein-Barr virus-containing B-cells for protection against MTX-induced inhibition of proliferation, and we have compared these requirements to those of 3 human malignant solid-tumor cell lines and the CCRF-CEM T-cell leukemia in culture across the range of dThd and Hyp concentrations found in humans. In order to obtain results that reflected, as closely as possible, the cellular pharmacology of primary human tumors, we have used cells recently cultured from human tumor xenografts growing in nude mice as well as several more established cell lines. A preliminary report of this study has been presented (47).

MATERIALS AND METHODS

Measurement of dThd and Hyp. Blood samples were obtained from normal subjects and patients with nonlymphomatous solid tumors at random times. dThd was measured in serum separated from formed elements as soon as clotting was complete. Hyp was measured in plasma from blood which had been drawn into precooled tubes, anticoagulated with EDTA, and separated immediately from formed elements by centrifugation at 4°C. Serum and plasma samples were stored at −30°C, at which dThd and Hyp were stable, until analyzed. dThd and
Hyp were quantitated by high-pressure liquid chromatographic assays, details of which have been described elsewhere (21, 57). For both assays, samples were prepared for analysis by precipitation of protein with 0.1 volume of 4.4 N perchloric acid, followed by clarification by centrifugation, and neutralization with Alamine/Freon TF as described by Khym (30). A high-pressure liquid chromatograph (Waters Associates, Milford, Mass.) with a reverse-phase C18/FiBondapak column was used for all determinations. Column output was monitored at 254 and 280 nm, and peak heights and areas were electronically integrated. Peaks were identified by retention time, absorbance ratios at 254 and 280 nm, enzymatic shift, and cochromatography of standards (32). For measurement of dThd, 100 µl of sample were injected, and the column was eluted with a 15-min linear gradient, starting with a 5 mm potassium phosphate buffer (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. For measurement of Hyp, 20 µl of sample were injected, and the column was eluted isocratically with 50 mm potassium phosphate buffer (pH 4.60). The lower limit of sensitivity for dThd was 0.04 µM and for Hyp, 0.1 µM.

Cell Types. Human bone marrow was obtained from patients undergoing orthopedic procedures and patients with nonhematological cancers not involving marrow by aspiration and was anticoagulated with preservative-free heparin. Peripheral blood T-cells were recovered from blood drawn by venipuncture from hematologically normal subjects by sedimentation on a Ficoll/Hypaque gradient (Ficoll/Paque; Pharmacia Fine Chemicals, Piscataway, N. J.). The 4 B-cell lines used in these studies were established from patients with mononucleosis or by in vitro Epstein-Barr virus transformation; these lines are described elsewhere: WI-L2 (37); SB (35); B.L.2 (52); and 894 (7). The CCRF-CEM line is a T-lymphoblastoid cell established from a patient with acute lymphoblastic leukemia (12). Three solid-tumor cell lines [an astrocytoma (T24), a melanoma (T242), and a colon carcinoma (T219)] were obtained from Dr. Hideo Masui of the Nude Mouse Research Center of the University of California, San Diego, Calif. All 3 of these lines were very recently established in culture directly from human tumor xenografts growing in nude mice using techniques outlined elsewhere (41). All cell lines were maintained in log-phase growth by serial passage in vitro in Roswell Park Memorial Institute media containing 10% extensively dialysed fetal calf serum (Flow Laboratories, Inglewood, Calif.). dThd and Hyp concentrations in the dialysed fetal calf serum were below the limits of detection when measured by high-pressure liquid chromatography.

Colony-forming Assays. The cytotoxicity of MTX was quantitated for human marrow CFU-GM and peripheral blood T-cells by colony survival in drug-treated cultures compared to cultures without drug exposure. CFU-GM were grown using a modification of the technique of Iscove et al. (29), described in detail elsewhere (23). The final culture contained 0.8% methylcellulose, a-medium without nucleosides (Grand Island Biological Co., Grand Island, N. Y.), 15% fetal serum (Flow Laboratories), and 20% leukocyte-conditioned medium, prepared according to the method of Iscove et al. (29). The concentration of Hyp and dThd in the final media were determined by high-pressure liquid chromatography to be 12 µM and <0.04 µM, respectively. Triplicate 1-ml cultures were incubated at 37° in 7.5% CO₂, and aggregates of greater than 40 cells were scored as colonies on Days 7 to 10 with an inverted microscope.

Peripheral blood T-colony-forming units were cultured in a-medium without nucleosides containing 0.8% methylcellulose, dialized 10% fetal calf serum with dialyzed PHA-conditioned medium at a final concentration of 20% (23), and PHA (Burroughs Wellcome Company, Research Triangle Park, N. C.) at a final concentration of 2 µg/ml. Aggregates of greater than 40 cells were counted as colonies on Day 7. The composition of colonies for both CFU-GM and T-cells was assayed by aspirating individual colonies with fine capillary pipets, pooling the cells, and preparing cytospins stained with Wright-Giemsa, α-naphthyl-sesterase, or chloroacetate esterase and by rosette formation with sheep RBC.

All assays were conducted in the continuous presence of 10 µM MTX, with variable initial concentrations of dThd in the presence of excess (10 to 100 µM) Hyp or variable concentrations of Hyp in the presence of excess (1 to 10 µM) dThd.

Growth Rate Assay. Cells in the log phase of growth were cultured for 72 hr in media containing 10% dialyzed fetal calf serum with 10 µM MTX and variable concentrations of dThd and Hyp as outlined for the colony formation assays above. The cytotoxicity of MTX was quantitated as reduction in steady-state growth rate relative to cultures without MTX but with the same concentrations of dThd and Hyp. Peripheral blood T-cells from normal donors were purified and cultured with PHA in a final concentration of 1 µg/ml as described elsewhere (3).

RESULTS

Charts 1 and 2 show the serum dThd and plasma Hyp concentrations, respectively, in samples obtained at random times from normal subjects and patients with solid tumors other than lymphoma. A wide range of values for both dThd and Hyp was observed in normal subjects and cancer patients. Among normal subjects with measurable levels, the geometric mean dThd concentration was 0.13 µM (range, <0.04 to 0.78 µM), and among cancer patients, it was 0.2 µM (range, <0.04 to 0.87 µM). The mean Hyp concentration in normal subjects was 0.51 µM (range, 0.2 to 1.9 µM), and in the cancer patients, 0.9 µM (range, 0.2 to 2.6 µM). The means and ranges for dThd are
was due to the fact that Hyp is rapidly released from the formed very close to those reported by other investigators using a the selection of appropriate dThd and Hyp concentrations to serum values are artifactually elevated. The average dThd and elements of the blood during the clotting process (57) so that by other investigators for serum Hyp (42). This discrepancy quantitated using a high-pressure liquid Chromatographie assay with a lower limit of sensitivity of 0.1 /IM.

Chart 2. Hyp concentration in plasma samples drawn at random times from normal subjects and patients with nonlymphomatous solid tumors. Blood was drawn into precooled tubes and anticoagulated with EDTA, and the formed elements were separated from plasma immediately by centrifugation. Hyp was quantitated using a high-pressure liquid chromatographic assay with a lower limit of sensitivity of 0.1 /IM.

very close to those reported by other investigators using a radioimmunoassay for dThd (18). However, the means for Hyp are significantly lower than the values of 13 to 16 /IM reported by other investigators for serum Hyp (42). This discrepancy was due to the fact that Hyp is rapidly released from the formed elements of the blood during the clotting process (57) so that serum values are artifically elevated. The average dThd and Hyp concentrations found in vivo provided reference points for the selection of appropriate dThd and Hyp concentrations to be tested against cultured human cells.

Normal and malignant cells were cultured in the presence of 10 /IM MTX, a serum concentration readily achieved in patients receiving high-dose MTX infusions (48), and either increasing concentrations of dThd in the presence of excess (1 to 10 /IM) dThd, or graded concentrations of dThd and excess (10 /IM) Hyp. The PC50 of dThd or Hyp was determined by extrapolation. Each PC50 value is the mean from a minimum of 3 experiments.

Table 1
PC50's of dThd and Hyp required to reduce the toxicity of 10 /IM MTX

<table>
<thead>
<tr>
<th>Assay type</th>
<th>Cell type</th>
<th>dThd</th>
<th>Hyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony survival</td>
<td>CFU-GM</td>
<td>0.15</td>
<td>NEa</td>
</tr>
<tr>
<td>Peripheral blood T-cells</td>
<td>0.2</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Growth rate</td>
<td>PHA-stimulated peripheral blood T-cells</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>B-cell lines</td>
<td>WI-L2</td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>957</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>894</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Solid tumor lines</td>
<td>Astrocitaryoma</td>
<td>0.7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Colon carcinoma</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>T-cell leukemia</td>
<td>0.7</td>
<td>7</td>
</tr>
</tbody>
</table>

a Concentration in 1uM/liter.
b NE, not measurable.

Chart 3. dThd dose-response curve for the protection of human CFU-GM (•) and peripheral blood T-cells (○) against the toxicity of 10 /IM MTX in the presence of 10 /IM Hyp. Each point represents the mean of triplicate plates from 4 colony survival assays performed with bone marrow or peripheral blood from separate donors; bars, S.E.

what lower dThd PC50 values. The full dThd dose-response curves for these 2 cell types are shown in Chart 3. It is of interest that the dThd PC50 for CFU-GM and normal T-cells and the Hyp PC50 for T-cells fall well within the range of the endogenous dThd and Hyp concentrations found in vivo.

Each of the cell types other than marrow CFU-GM was tested for the ability of excess dThd to reduce MTX toxicity in the presence of physiological concentrations of Hyp, and each of the cell types including marrow CFU-GM was tested for the ability of excess Hyp to reduce MTX toxicity in the presence of physiological concentrations of dThd. In neither situation was a significant degree of protection afforded to any cell type tested. Although the dThd and Hyp concentration in the microenvironment of the tumor may differ from the average serum values, this result indicates that, in contrast to some murine cell types (4-6, 43, 50), all of the human cell types tested in this study (with the possible exception of marrow CFU-GM which could not be tested) required both dThd and Hyp for
protection against MTX and that there were no cells in which MTX produced exclusively an antipurine or antithymidylate effect.

Charts 4 and 5 compare the dose-response curves for dThd and Hyp protection for marrow CFU-GM (dThd only), a representative B-cell line, and the 3 malignant solid-tumor lines. Although there was some scatter in the PC₀₀ values, the shapes of these curves were remarkably similar with respect to slope despite the fact that a colony-forming assay was used for marrow, whereas growth rate assays were used for the cell lines. All were steep enough in their mid-portion so that changes in dThd of Hyp concentration over the naturally occurring range found in normal subjects and cancer patients (Charts 1 and 2) could produce large changes in MTX-induced inhibition of proliferation.

dThd and Hyp concentrations were measured in the plasma recovered in bone marrow aspirated from 14 patients undergoing diagnostic marrow examinations (some of whom were taking allopurinol) to determine whether the concentrations of these salvage pathway metabolites in the marrow microenvironment differed significantly from those in venous plasma. No dThd was measurable in any of the marrow aspirates examined (<0.04 μM). In contrast, Chart 5 shows that the average Hyp concentrations of 11 μM in marrow were 40-fold higher than the average in normal plasma and 12.5-fold higher than that in cancer patients. In the 8 cases where simultaneous venous and marrow Hyp concentrations were measured, the Hyp concentration in the marrow averaged 6.1 μM, 4.2-fold higher than plasma (p < 0.001, t test for paired observations) (Chart 6). To exclude the possibility that hemolysis artifactually elevated the marrow Hyp concentrations, Hyp was measured in plasma from blood samples subjected to extensive mechanical hemolysis and compared to unhemolysed controls from the same subject. No differences in Hyp concentration were found. In cultures of marrow, it was found that 10 μM Hyp was sufficient to make CFU-GM dependent only on the availability of dThd (Chart 3). Thus, these results suggest that, in vivo, the local concentrations of purine metabolites are high enough that the marrow toxicity of MTX is due entirely to its antithymidylate rather than its antipurine effect.

DISCUSSION

Although the ability of dThd and Hyp to protect cells against MTX has been widely studied, all prior investigations have used either murine cells (4–6, 16, 25–27, 50) or long-established human tissue culture cell lines (11, 16, 43, 50) whose cellular pharmacology may be quite different from normal and malignant human cells in vivo. In this study, by using freshly aspirated human marrow and cell lines recently established from human carcinoma xenografts, as well as more established cell lines, we have attempted to reduce the problems associated with extrapolating in vitro data to the in vivo situation while still using conditions that permit control of salvage pathway metabolite concentration.

Based on their requirements for protection, the results of this study indicate that MTX inhibits both de novo purine synthesis and thymidylate synthesis in all of the cells tested. However, recognizing the limitations of extrapolations from in vitro data, differences between the degree of inhibition of thymidylate versus purine synthesis in marrow and in the malignant cells do not appear to be large enough to permit substantial improvement in therapeutic ratio through the use of combinations of MTX with dThd or purine in vivo. Although dThd, in the presence of excess Hyp, provided protection for marrow CFU-GM at concentrations 5–7-fold lower than those required by the
malignant cells studied, the significance of this difference is diminished by the wide variability of the serum dThd concentration in vivo. The range of endogenous serum dThd concentrations found in this study, and reported by others (18), spans nearly the full amount of the difference between the dThd PC$_{50}$ for marrow CFU-GM and for the malignant cells. As suggested by the wide scatter of serum dThd concentrations measured in randomly drawn samples, the dThd concentration in the same subject varies from a minimum of 2-fold to more than 6-fold over a 24-hr period.  

A similar range of variation has been observed in patients receiving constant infusions of dThd (8 g/sq m/day) even when constant infusion pumps were used for administration (10, 20–22). This makes it very improbable that serum dThd concentrations could be regulated closely enough in vivo to take advantage of only a 5- to 7-fold difference in dThd PC$_{50}$, particularly since large doses of MTX themselves decrease the endogenous serum dThd concentration.  

The low response rates observed to date in several clinical trials of MTX in combination with dThd tend to support this conclusion (10, 20, 21, 24).

Although we were not able to determine the Hyp PC$_{50}$ for CFU-GM, the relatively narrow span of Hyp PC$_{50}$ values for the other cells examined in this study and the wide scatter of plasma Hyp concentrations found in randomly drawn samples suggest that improvement in MTX selectivity through modulation by Hyp in the presence of excess dThd may be equally difficult. Since the microenvironment of the marrow already appears to contain a high enough Hyp concentration to make marrow protection against MTX mainly a function of dThd availability (see below), additional purine administration would not be expected to improve the therapeutic ratio. However, it has been established in the murine L1210 leukemia that the therapeutic ratio of MTX can be improved by combining it with dThd and a source of purines for malignant cells that are severely deficient in purine salvage pathway enzymes (8). Thus, human tumors that are highly resistant to 6-mercaptopurine or 6-thioguanine may not only be collaterally sensitive to MTX, but their Hyp PC$_{50}$ values may be sufficiently different from marrow to permit enhanced selectivity when dThd and Hyp are administered concurrently.

It has been reported that T-cells are relatively deficient in dThd-catabolic enzymes compared to B-cells (9, 13, 19). In this regard, it was of interest to note that the dThd PC$_{50}$'s for both PHA-stimulated normal T-cells and the T-cell leukemia CCRF-CEM were lower than the dThd PC$_{50}$ values for all of the B-cells examined. This may reflect more effective utilization of salvaged dThd by T-cells whose capacity to degrade dThd is limited.

The effect of variations in local or systemic salvage metabolite concentration is amplified by the fact that, for all of the cells examined, the dose-response curve for antimetabolite protection was relatively steep. In culture, small changes in dThd and Hyp concentrations resulted in large differences in the degree of MTX toxicity. Thus, since the mean serum dThd concentration falls near the mid-portion of the dThd dose-response curve, fluctuations in serum dThd concentration may account for some of the notorious variability in the clinical toxicity of MTX in vivo (17). The finding that all the human cells investigated required both dThd and Hyp for protection against MTX would appear to be at odds with the clinical experience in humans, which indicates that patients can be protected against myelosuppression from otherwise lethal doses of MTX with dThd doses that increase average serum dThd concentrations as little as 2-fold (21) and that doses of dThd that raise the serum dThd concentration to 1 μM nearly completely block MTX toxicity without the need for administration of purines (10, 20–22). The finding of high local concentrations of Hyp in bone marrow provides a reasonable explanation for this apparent paradox, since in the presence of excess Hyp the dose-response curve for dThd protection of CFU-GM was steep, and complete protection was achieved at a dThd concentration of 1 μM. Since Hyp also decreases the rate of de novo purine synthesis (53), high marrow concentrations of Hyp may also explain earlier observations, based on differential labeling of pyrimidine and purine bases in DNA by [14C]formate, that purine synthesis in marrow cells was less dependent on the de novo pathway than pyrimidine synthesis (11, 34, 54, 55). The source of marrow Hyp is unknown, but catabolism of nucleic acids from senescent cells is a probable contributor. Tumor nodules may also contain high enough local concentrations of dThd or Hyp to protect themselves against the antipurine action of MTX, particularly since hypoxia induces catabolism of purine ribotides to Hyp (1, 2, 38, 44).

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