Activity of Thymidine as a Chemotherapeutic Agent against Human Tumor Xenografts in Nude Mice

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ABSTRACT

The chemotherapeutic activity of thymidine (dThd) was tested against four human tumor xenografts growing in nude mice, including a melanoma, an oat cell carcinoma of the lung, a colon carcinoma, and a breast carcinoma. Tumor-bearing mice were given an infusion of dThd (1 g/kg/day) s.c. for 72 hr each week for three weeks. Tumor growth in the treated mice was compared to that in randomized concurrent control mice infused with media alone. A significant effect was found only for the melanoma, and it was cytostatic rather than cytotoxic. Even when melanomas of very small initial volume were treated, there were no complete regressions, and tumor growth resumed when dThd treatment was stopped. In culture, sustained dThd concentrations of >3.2 mM were required to cause death of the melanoma cells; in the mice the dThd level during infusion ranged from 1 to 5 mM. This exposure to dThd, although failing to produce a tumor response, did produce significant toxicity in the nude mice in the form of myelosuppression and leukopenia. Flow cytometric analysis of marrow cells during the dThd infusion showed an accumulation of cells in S phase, but proliferation was not completely halted since cells with G2-M content of DNA were present in the marrow even after 72 hr of dThd exposure. This study failed to demonstrate a therapeutically useful effect of dThd on these tumors.

INTRODUCTION

The ability of excess dThd to reversibly inhibit proliferation of cells in culture has been known for many years, and this technique is widely used to synchronize cell populations (4, 6, 20, 21, 26). The arrest of replication has been attributed to inhibition of ribonucleotide reductase by dTTP, formed from dThd intracellularly, which causes depletion of the dCTP pool to the point where DNA synthesis is impaired (3, 16–18, 22, 26). Several recent reports indicate that malignant cells may be more sensitive to the lethal effect of dThd than are normal cells (14, 25), which is consistent with earlier suggestions that malignant cells are less capable of maintaining viability under adverse growth conditions (5, 19). In particular, Lee et al. (14) reported that human melanoma and colon carcinoma cells in culture are more sensitive to dThd than are normal melanocytes and intestinal epithelial cells. These investigators subsequently reported that continuous infusions of very large quantities of dThd for 72 hr each week could inhibit the growth of human melanomas and several other human tumor xenografts growing in nude mice without causing recognizable toxicity (13, 15). This observation suggested that dThd might be a useful chemotherapeutic agent against human melanoma, a tumor for which there are few clinically effective drugs. The apparent lack of dThd toxicity in the nude mouse indicated the potential of a favorable therapeutic ratio, and Phase I-II clinical trials in humans have been initiated.

We have examined the activity of dThd against four human tumor xenografts in nude mice: a melanoma, an oat cell carcinoma of the lung, a colon carcinoma, and a breast carcinoma. In this study, we confirmed a significant cytostatic effect of dThd on the growth of the melanoma but found only a minor effect of dThd on the oat cell carcinoma, and no effect on the growth of the colon and breast carcinomas. In addition, we found that dThd produced significant toxicity in the form of myelosuppression.

MATERIALS AND METHODS

Tumors and Mice. All 4 tumors were established by inoculation of fresh biopsy material directly from patients in nu/nu mice on a BALB/c background bred at the Nude Mouse Research Center, University of California, San Diego. The tumors had been maintained by serial s.c. passage for less than 3 years. The number of previous passages ranged from 11 to 19 at the time of study: the average passage times were 44 days for the melanoma (T242), 75 days for the oat cell carcinoma (T293), 60 days for the colon adenocarcinoma (T183), and 81 days for the infiltrating ductal carcinoma of the breast (T112). The breast cancer contained estrogen receptor protein and grew only in mice in whom a 25-mg pellet of estrogen was placed one day after tumor inoculation. Mice were housed in plastic cages with filter tops at 26°C. The mice were given nonsterile water and chow and kept in a clean environment. T242 melanoma cells were established in culture directly from a biopsy of the tumor xenograft in nude mice by H. Masui, Nude Mouse Research Center, University of California, San Diego. These cells were grown in autoclavable Eagle’s minimal essential media (Flow Laboratories, Inc., Inglewood, Calif.) supplemented with 10% fetal calf serum.

Treatment Program. Experiments were initiated by giving groups of mice s.c. inoculations in the flank with equal volumes of tumor brei made by mincing of tumor fragments. When the tumors reached approximately 5 x 5 mm, pairs of animals with equal-sized tumors were randomized to receive either dThd suspended in Dulbecco’s minimal essential media or media alone. dThd obtained from the National Cancer Institute was dissolved in media at 40 mg/ml, the pH was adjusted to 7.4, and the solution was sterilized by filtration. The dThd dose of 1 g/kg/hr was delivered in a volume of 0.51 ml/hr through a...
catheter placed s.c. over the back of the unrestrained mouse, by using a constant infusion pump. Mice were infused for 72 hr each week for 3 weeks, thus receiving dThd on Treatment Days 1 to 3, 8 to 10, and 15 to 17. Animal weights and the minimum and maximum tumor diameters obtained by caliper measurement were determined twice weekly. Tumor volumes were estimated by using the formula \( V = \pi ab^2/8 \), where \( a \) is the maximum diameter and \( b \) is the minimum diameter. Each tumor was scored as demonstrating growth or no growth during the treatment period.

**Plasma dThd and Thymine Measurements.** Blood obtained by decapitation was anticoagulated with heparin, and after removal of formed elements by centrifugation, the plasma was acidified with 0.1 volume of 4.4 M perchloric acid, then clarified by centrifugation, and neutralized with Alamine/Freon TF as described by Khym (12). The neutralized samples were then chromatographed by high-pressure liquid chromatography with an RP-18 column and developed with a 5 mM potassium phosphate, pH 3.34, acetonitrile gradient (8).

**Flow Cytometry.** Femoral marrow was expressed from the bone by using a 23-gauge needle and washed in 0.9% NaCl solution, and cell counts were performed on a Model ZBI Coulter counter. Marrow cells were fixed in 70% ethanol in water, and stored at 4°C until analysis by flow cytometry. Fixed cells were stained with ethidium bromide (10 mg/ml) and mithramycin (25 mg/ml) (2), and analyzed with a Phywe ICP 22 flow cytometer. The proportion of cells in each phase of the cell cycle was determined by using the approximation method of Holley and Kiernan (9) to obtain estimates of the fraction of cells in G1-G0, S, and G2-M phases of the cell cycle. Chicken cells were added as an internal standard (1, 23).

**RESULTS**

**Tumor Growth Rates.** Charts 1 to 4 show the patterns of growth for the control and dThd-treated groups for each of the 4 tumors studied. Tumor volumes are shown for each animal during the 17-day treatment period and for 43 days thereafter. Each tumor was scored for growth or no growth during the 17-day treatment period, and the number of tumors in each category is shown in Table 1. Abrupt decreases in tumor volume are indicative of ulceration of the tumor through the skin with subsequent necrosis, rather than true tumor regression.

The melanoma was the most rapidly growing of the 4 tumors studied. There were 11 mice in the control group and 12 in the dThd-treated group. All 11 control tumors showed progressive growth during the treatment period, whereas only 5 of the 12 in the treatment group showed progressive growth during this period, a difference which is significant (\( p = 0.037 \)). For the oat cell carcinoma of the lung, there were 8 animals in the control group and 7 in the treatment group, and although there appears to be some downward and rightward shift of the growth curves, the difference in the proportion of mice whose tumors grew during the treatment period was not statistically significant. In the case of the colon carcinoma, there were 10 mice in both the control and dThd treatment groups, and all 10 animals in each group demonstrated progressive growth. Assessment of the sensitivity of the breast adenocarcinoma to dThd was hampered by a large number of spontaneous regressions in both groups. There were a total of 24 animals bearing the breast carcinoma, 10 in the control group and 14 in the treated group. Three animals were identified in the control group with progressive tumor growth during the treatment period, and no animals in the treated group, but this was not a
Antitumor Activity of dThd

There was no complete regressions that could be ascribed to treatment, and in surviving hosts most tumors regrew following discontinuation of weekly dThd treatment.

Effect of Tumor Volume. A cytostatic effect of dThd was observed against the melanoma when the mean initial tumor volume was 247 ± 124 (S.D.) μl, representing tumors of approximately 6 mm in diameter (Chart 1). To determine whether a cytotoxic effect of dThd could be demonstrated, in another group of mice treatment was started one day after tumor inoculation, at which time the mean tumor volume was 10.8 μl in the control group and 10.3 μl in the treatment group, reflecting a tumor diameter of about 2.0 mm. Chart 5 shows that dThd did suppress tumor growth during the treatment period; the mean tumor volume of 45.2 ± 26 μl in the treatment group at the end of dThd exposure on Day 17 was significantly smaller than the mean tumor volume of 115 ± 49 μl in the control group (p < 0.01). However, even with these very small initial tumor volumes, 3 weekly courses of dThd failed to eradicate any tumors, and progressive growth occurred in all surviving hosts following the treatment period.

Table 1

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>Tumor type</th>
<th>Control growth</th>
<th>dThd growth</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>T242</td>
<td>Melanoma</td>
<td>11/11</td>
<td>5/12</td>
<td>0.037</td>
</tr>
<tr>
<td>T293</td>
<td>Lung (oat cell)</td>
<td>7/8</td>
<td>5/7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>T183</td>
<td>Colon</td>
<td>10/10</td>
<td>10/10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>T112</td>
<td>Breast</td>
<td>3/10</td>
<td>0/14</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

* Number of mice demonstrating tumor growth during treatment period/number of mice in group.

significant difference. There was also no significant difference between the 2 groups with respect to the number of breast carcinomas that resumed growth after showing no growth or regression during the treatment period. Overall, where an effect of dThd treatment was detectable, it appeared to be cytostatic rather than cytotoxic.


Chart 5. Growth of T242 melanoma with initial volume of 10 μl. Treatment was started one day after inoculation of tumor: A, control; B, dThd treated. Arrow, end of 17-day treatment period; bar, animal death.
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In Vitro Sensitivity of Melanoma Cells. Melanoma cells established in culture directly from biopsies of the xenograft in the nude mice were assayed for their sensitivity to a 72-hr exposure to dThd. Untreated cultures had a doubling time of 2.5 days. The cytostatic concentration of dThd was determined to be 1.4 mM. After a 72-hr exposure to 1 mM dThd, the melanoma cells demonstrated some ability to recover when dThd was removed and resumed growing at 43% of the rate of control cultures. However, a 72-hr exposure to either 3.2 mM dThd, which killed 36% of the cells, or to 10 mM dThd, which killed 56% of the cells, completely prevented resumption of growth. This result suggests a steep dose response for dThd in this concentration range. In contrast to dThd, thymine in concentrations < 10 mM was completely nontoxic.

Toxicity. Neither the control nor the dThd-treated mice demonstrated visible evidence of toxicity. Three courses of dThd treatment did not cause weight loss, and surviving mice remained healthy until their tumors became very large. Deaths that could not be attributed to anesthesia or infection occurred in both treated and untreated groups. There were 4 such deaths among the 47 control mice, and 10 among the 51 dThd-treated mice (p > 0.05). No visible pathology was found on gross examination of the animals that died. During the infusion, all mice became bloated with s.c. fluid, and these otherwise unexplained deaths were attributed to fluid overload.

To assess the effect of dThd on normal nude mouse marrow, the peripheral blood leukocyte count and total number of cells per femur were determined daily during the 72-hr infusion and for 4 days thereafter. In addition, flow cytometric analysis was performed to monitor changes in the marrow cell cycle distribution. Chart 6 shows that the number of marrow cells per femur decreased continuously during the infusion of dThd. A nadir of 38% of the initial cellularity was reached between Days 3 and 4, following which there was a prompt recovery. The peripheral blood leukocyte count decreased in a similar manner, reaching a nadir of 20% of the initial value on Day 2.

Chart 7 shows the changes in the distribution of marrow cells in various phases of the cell cycle that occurred during and following a 72-hr infusion of dThd. There was a progressive accumulation of cells in S phase, starting from 20.2% at the beginning of the infusion and reaching 37.7% at 72 hr. This accumulation of cells appeared to be primarily at the expense of the proportion of cells in G2-M, which was initially 71.5% and had fallen to 59.2% by the end of the 72-hr infusion. It is interesting to note that, although the proportion of cells in G2-M fell from 8.2% to 3.1%, it did not decrease to 0, suggesting that some undetermined proportion of marrow cells continued through S phase and entered G2-M despite the presence of greater than 1 mM dThd. By 24 hr after the end of the infusion, there was an increase in the fraction of cells in G2-M and a further decrease in the fraction of cells in G2-M, consistent with the movement of a cohort of cells from G, through S into G2-M as the plasma level of dThd fell rapidly to pretreatment concentrations.

dThd and Thymine Plasma Levels. Chart 8 shows that, during s.c. dThd infusion, dThd plasma levels were in the range of 1 to 5 mM, and thymine levels were in the range of 0.1 to 0.7 mM. Near steady-state concentrations of dThd and thymine were achieved by 6 hr and had returned to pretreatment levels by 24 hr after the end of the s.c. infusion. The endogenous dThd level of 6.3 nM indicated in Chart 8 is a maximal estimate since in nude mouse plasma the dThd, when present in very low concentrations, could not be completely resolved from a small interfering peak by high-pressure liquid chromatography.

DISCUSSION

This study was undertaken in an effort to confirm the reports of Lee et al. (13, 15) that dThd could both suppress the initial growth of human melanoma xenografts in nude mice (13) and cause regression of established tumors (15). Since it was reported that dThd could cause regression of several other types of human malignancies (Ref. 15 and Footnote 4), we tested dThd against a breast and a colon carcinoma and an oat cell carcinoma of lung in addition to a melanoma by using the same dose schedule used by Lee et al. (13). The results indicate that there was some cytostatic effect of dThd on the initial growth of the melanoma, which was the most rapidly growing of the 4 tumors studied. Although there was a suggestion that dThd slowed the growth of the oat cell carcinoma, this

Chart 6. The effect of a 72-hr infusion of dThd (1 g/kg/hr) on the peripheral blood leukocyte count and bone marrow cellularity in nude mice. Bars, S.E.

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did not reach statistical significance, and no effect was evident on the other 2 tumors. Where an effect of dThd was detectable, it appeared to be cytostatic rather than cytotoxic. Although some partial regressions occurred, there were no complete regressions even of the melanoma and even when very small melanomas with diameters of 2 mm were treated with 3 courses of dThd starting one day after tumor inoculation. All tumors grew progressively as soon as the dThd therapy was withdrawn. These results indicate that the dThd dose schedule of 1 g/kg/hr for 72 hr for 3 courses was insufficient to rid the mice of even a small tumor burden.

The in vitro studies with the melanoma indicated that, whereas a 72-hr exposure to 1.4 mM dThd was sufficient to produce a cytostatic effect, concentrations of 3.2 mM or higher were required to produce cytotoxicity and to block immediate resumption of growth once the dThd was removed. During the mouse infusions, plasma dThd levels of 1 to 5 mM were established by 6 hr and maintained for the remainder of the 72-hr period of drug administration. Although these levels were only cytostatic in vivo, the steep dose response for dThd in this 1- to 10-mM range evident in vitro suggests that a cytotoxic effect might be achieved with somewhat higher plasma dThd levels. Because of the limited solubility of dThd and the poor tolerance of mice to infusion of larger fluid volumes, we were unable to test the effect of such higher plasma dThd concentrations. It is noteworthy that the dThd concentrations achieved in these mice are comparable to those achieved in humans with doses of 75 g/sq m/day (10). Although this indicates that this nude mouse model should be adequate from a pharmacological point of view to test the sensitivity of human tumors to this drug, since the in vivo dThd levels were only cytostatic, perhaps higher dose rates will also be required in humans to demonstrate an antitumor effect against melanoma.

In the nude mouse, we found that dThd infused at 1 g/kg/hr produced significant myelosuppression. There was a rapid reduction in bone marrow cellularity and peripheral blood leukocyte counts during the 72-hr infusion, with recovery starting promptly after dThd was discontinued. In confirmation of earlier reports (13), no other major toxicity was evident, but it was not specifically sought for in other organ systems. In other studies from this laboratory (10), dThd concentrations in excess of 1 mM were found to routinely block the proliferation of human granulocyte-macrophage colonies in vitro.

In some systems, the response of normal and malignant cells to high concentrations of dThd is qualitatively different (14, 24, 25). It has been reported that nonmalignant cells are able to avoid damage caused by dThd-induced pyrimidine triphosphate starvation by arresting their growth, whereas malignant cells continue to divide and are injured in the process (24). However, the persistence of a fraction of normal nude mouse marrow cells in the G2-M phase of the cell cycle throughout a 72-hr exposure to >1 mM dThd and the substantial myelosuppression that ensued indicate that, although proliferation may have been slowed, some marrow cells continued to cycle and were damaged. Failure of growth arrest was also observed with human B-cells and phytohemagglutinin-stimulated T-cells exposed to dThd in culture and leukemic T-cells exposed to dThd in vivo (Ref. 10 and Footnote 5). It is unlikely that dThd would produce clinically useful effects in the patients from which the 4 tumors included in this trial were obtained, and the results of this study suggest that the therapeutic ratio in humans may not be high due to myelosuppression. Nevertheless, dThd may demonstrate greater activity on another dose schedule, and it may be possible to take advantage of differential tissue sensitivities to dThd to selectively modulate the activation of other antimetabolites, such as 1-β-D-arabinofuranosylcytosine (7).

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