Antitumor Activity and Minimal Toxicity of Concentrated Thymidine Infused in Nude Mice

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ABSTRACT

To avoid infusing large volumes of fluid while treating patients with the standard thymidine solution (30 g/liter), it may be possible to administer this drug in more concentrated form. At 25°C, thymidine is saturating at a concentration of 52 g/liter of 0.6% NaCl solution, and the thymidine concentration at saturation increases with temperature. Nude mice were infused at 29°C with thymidine (60 or 72 g/liter) in cycles consisting of 4 to 5 days infusion followed by 9 days rest. Therapeutically effective doses of concentrated thymidine did not cause significant mortality in mice, and weight loss attributable to treatment was small and reversible. Significant growth inhibition of CA 1 human melanoma heterotransplants was observed after 3 treatment cycles. After 4 or 5 cycles, tumor responses were obtained in 7 mice (6 complete responses) of 12 inoculated with this tumor. These results show that concentrated thymidine solutions are highly effective against human tumor heterotransplants in nude mice and suggest that clinical use of concentrated thymidine may allow practical administration of maximum tolerated doses of this drug.

INTRODUCTION

Thymidine infused continuously at a concentration of 28.5 g/liter causes regression of human tumor xenografts implanted into nude (athymic) mice (18). Plasma levels of 1 to 2 mm of thymidine can be maintained in animals and humans with tolerable and reversible toxicity (2, 25). As a result of these findings, high-dose thymidine infusion was entered into clinical trial.

The antitumor effect of thymidine infused at a concentration of 30 g/liter has been most evident in patients with T-cell leukemia and lymphomas (1, 2, 4, 13, 15) and has been observed in some patients with solid tumors, although remissions have not been obtained in the latter cases (4). Toxicity, predominantly bone marrow depression, has been mild and reversible (1, 4, 15). These results suggest that greater antitumor effect could be obtained with tolerable toxicity by administering larger doses of thymidine. With prolonged infusion of very high doses of this agent, thymidine plasma levels of 3.8 to 5.5 mm of thymidine were attained with toxicities that were relatively well tolerated. Significant antitumor activity was achieved, including a temporary complete remission in a patient with refractory acute lymphoblastic leukemia (1).

Due to the rapid clearance of this nucleoside (13, 24), large volumes of 30 g/liter thymidine solution must be infused contin-

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2 To whom requests for reprints should be addressed.

3 J. S. Stehlin, Jr., P. D. de Ipolyi, and P. J. Greeff, unpublished observations. Received 3/22/83; revised 7/7/83, 11/12/84; accepted 12/18/84.

4 The abbreviations used are: saline-MEM, 50:50 solution of 0.9% NaCl solution/Eagle's minimal essential medium; HPLC, high-pressure liquid chromatography.

5 B. C. Giovanella, A. Lockshin, T. Kozielski, C. Quian, and J. S. Stehlin, Jr., manuscript in preparation.
ment were classified as complete responses. When at least 50% but not all of the tumor became necrotic, the response was considered to be partial.

RESULTS

Solubility. The concentration of thymidine at saturation was determined after heated, highly concentrated (300 g/liter) thymidine solutions were cooled to specific temperatures [±0.4°C (SD)]. Thymidine measurements were taken 2 to 10 days after copious precipitates had formed. Results of these determinations for thymidine dissolved in 0.6% NaCl solution are shown in Chart 1. At 25°C and 37°C, thymidine was saturating at concentrations of 52 and 78 g/liter, respectively. Concentrations at saturation were about 5% higher in saline-MEM, probably due to partial ionization of the weakly acidic nitrogen 3 of thymidine (pKₐ = 9.8) when the media became slightly alkaline during incubation. No precipitation occurred at 29°C in thymidine solutions (60 or 72 g/liter) prepared in saline-MEM. HPLC analysis showed that less than 0.1% of the thymidine was hydrolyzed to thymine after preparation and storage of these concentrated solutions at 29°C for 9 months.

Serum Thymidine and Thymine Concentrations. HPLC analysis of thymidine and thymine levels in mouse serum showed that the mean plateau of thymidine concentration increased from 1.3 to 2.5 mM when thymidine at 60 g/liter was infused in doses ranging from 62 to 115 g/kg/cycle (Chart 2). Mean plateau thymine concentrations in the same dose range were 0.6 to 0.7 mM. There were no significant differences in serum thymidine or thymine levels attained during the first infusion cycle compared to subsequent cycles. At a higher thymidine dose (156 g/kg/cycle), the serum levels of thymidine increased to nearly 8 mM.

Toxicity. Toxicity studies with more concentrated thymidine were first conducted in nude mice without tumors. The mice were infused with thymidine or saline-MEM for 1 to 5 cycles; each cycle usually consisted of 4 to 5 days continual infusion followed by about 9 days of rest. In most cases, thymidine was infused at a constant rate, although in some instances the infusion rate was increased during a cycle to obtain the desired total dose per cycle. In earlier experiments, saline-MEM was infused during rest periods at a rate of about 8 ml/day. After development of a new system of infusion which allows detachment of the animals from the infusion catheters, the mice were not restrained or infused during rest periods. Table 1 shows the tolerance of mice to concentrated thymidine for the range of doses (60 to 119 g thymidine/kg/cycle) used to treat tumor-bearing animals (see below). The basis for evaluating survival was if the test animal lived at least 10 days after completion of thymidine infusion. After 98 thymidine treatment cycles in this dose range, only 1 animal died. Greater mortality (3 deaths out of 9 cycles) was evident in animals given higher doses of thymidine (120 to 160 g/kg/cycle). Control untreated mice suffered 2 deaths after 54 cycles of saline-MEM infusion. The median survival of thymidine-treated mice was not significantly different from the control group.

\[ T. \text{Kozielski, C. Quian, B. C. Giovanella, A. Lockshin, and J. S. Stehlin, Jr., manuscript in preparation.}\]
CONCENTRATED THYMIDINE

Table 1

Effect of concentrated thymidine on weight and mortality of nude mice

Thymidine dissolved at 60 g/liter was infused in 96 of the treatment cycles, and thymidine at 72 g/liter was infused in 2 of these cycles. Saline-MEM was infused into control mice. Surviving animals lived at least 10 days postinfusion.

<table>
<thead>
<tr>
<th>First cycle</th>
<th>Second to fifth cycles</th>
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<tbody>
<tr>
<td></td>
<td>No. of mouse treatments</td>
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<td>Control (untreated)</td>
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<tr>
<td>Thymidine dose (g/kg/cycle)</td>
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<tr>
<td>60–69</td>
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<td>90–99</td>
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</tr>
<tr>
<td>100–119</td>
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</tr>
<tr>
<td>Total at thymidine dose of (g/kg/cycle)</td>
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</tr>
</tbody>
</table>

* Mean ± SD.

** Weight losses did not differ significantly with dose in this range of doses.

Table 2

Effect of concentrated thymidine on weight and mortality of tumor-bearing nude mice

Thymidine dissolved at 60 g/liter was infused in 47 of the treatment cycles, and thymidine at 72 g/liter was infused in 8 of these cycles. Saline-MEM was infused into control mice. Surviving animals lived at least 10 days postinfusion. The 2 treated mice and 1 control mouse that died were implanted with the CA 1 melanoma.

<table>
<thead>
<tr>
<th>First cycle</th>
<th>Second to fifth cycles</th>
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<tbody>
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<td>No. of mouse treatments</td>
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<td>Control (tumor-bearing untreated mice)</td>
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</tr>
<tr>
<td>Thymidine dose (g/kg/cycle)</td>
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<td>3</td>
</tr>
<tr>
<td>Total at thymidine dose of (g/kg/cycle)</td>
<td>22</td>
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</tbody>
</table>

* Mean ± SD.

** Weight losses did not differ significantly with dose in this range of doses.

from control mice infused with the same number of cycles and was not dependent on the amount of thymidine infused within the range of doses shown in Table 1. The median survival posttreatment for 8 mice infused with thymidine for 4 to 5 consecutive cycles was 192 days compared to 183 days for mice infused with saline-MEM. No unusual pathology attributable to thymidine treatment was evident by autopsy or histopathological examination. There was no evidence of kidney damage caused by crystals of thymine, which is considerably less soluble than thymidine (4, 11).

The mice tolerated at least 5 consecutive cycles of thymidine treatment without irreversible toxicity as judged by weight loss. The greatest weight loss occurred during the first cycle for both thymidine-treated and saline-MEM infused mice. Essentially, all the weight was regained during the rest portion of the cycle if the mice were detached from the infusion apparatus. By the start of the third cycle, the net weight loss was 1.4 ± 3.3% for treated and 0.7 ± 8.4% for untreated mice. There was no correlation between weight loss and the amount of thymidine infused for the dose range of 60 to 119 g thymidine/kg/cycle.

The tolerance to concentrated thymidine of mice bearing human tumor xenografts (mainly the CA 1 melanoma) was also tested (Table 2). These data were compiled for mice bearing tumors which had been implanted 60 days or less prior to starting treatment and which did not regress during or immediately after thymidine infusion. Mortality was not significantly different for treated compared to untreated control tumor-bearing mice. The weight loss of these 2 groups of mice was comparable and was not dependent on the thymidine dose within the limits of 60 to 119 g thymidine/kg/cycle.

Antitumor Effects. The antitumor effect of concentrated thymidine was tested in 3 separate experiments with mice bearing the CA 1 melanoma. In each experiment, equal numbers of treated and untreated animals were used. In total, 12 animals were infused for up to 5 cycles at doses no higher than 87 g/kg/cycle for the first cycle and 109 g/kg/cycle for subsequent cycles, i.e., at well-tolerated doses (Tables 1 and 2), and another 12 animals were infused with comparable volumes of saline-MEM. The effect of repeated infusions of concentrated thymidine or saline-MEM on the growth of the CA 1 melanoma in one group
of 8 mice is shown in Chart 3. Growth inhibition of implanted tumors by thymidine, as measured by increases in tumor volume relative to the initial volume, began to be evident by the third cycle but was not significant statistically \(P < 0.05\) until the start of the fourth cycle. In 7 of the thymidine-treated animals, the tumors responded (6 completely) whereas all control animals died with massive tumors (median survival, 115 days postinoculation). The doses given to each responding animal are shown in Table 3. Three animals infused with thymidine died before completing 5 cycles, as did 2 untreated mice. A third control mouse died shortly after the fifth saline-MEM cycle was completed. These deaths of thymidine-treated and untreated animals probably were tumor related. Responses to concentrated thymidine (72 g/liter in one case) were also observed in a few mice inoculated with a human breast carcinoma or with FO 1 melanoma cells.

With effective thymidine treatment, tumor response usually was noted first as a selective blackening of the tumor, which became pulpy and appeared grossly necrotic. Often the host became sickly and sometimes expired, apparently due to resorption of large amounts of degenerating tumor tissue. In animals that survived the sloughing and resorption of necrotic tumor, a clean scar was formed at the former tumor site (Fig. 1). No such process has been observed with untreated mice bearing the CA 1 melanoma xenograft or the other tumors tested. Overall, 6 of 11 (55%) of the mice survived cycles which led immediately to massive response. The animals that died during tumor necrosis had received immediately beforehand doses of thymidine (range, 65 to 106 g/kg/cycle) which were well tolerated by non-tumor-bearing or by nonresponding mice.

### DISCUSSION

It is a current misconception that the maximum solubility of thymidine is 30 g/liter (2). At 25° C, the solubility at saturation is substantially higher, 52 g/liter when dissolved in 0.6% NaCl solution. Like many compounds, the aqueous solubility of thymidine rises rather steeply with increased temperature (Chart 1). Other investigators have infused rodents with thymidine dissolved at 50 g/liter (5, 14), and we infused mice with thymidine at 60 to 72 g/liter. With gentle warming, even higher concentrations might be used. Because of the stability of this nucleoside, thymidine solutions can be heated and then stored for long periods without refrigeration. These properties allow practical use of thymidine at substantially higher concentration and with less fluid than has been used to date clinically. We therefore conducted studies to determine if human tumor xenografts respond to concentrated thymidine at doses tolerated by the nude mice hosts.

The toxicity data accumulated to date show that Swiss nude mice readily tolerate repeated infusions of concentrated thymidine at doses which increase the serum thymidine concentration to about 1 to 3 mM. There was no significant mortality for mice treated with these doses of thymidine, whether the animals were tumor free or bore tumors which did not necrotize during or immediately after thymidine treatment. Moreover, repeated cycles of thymidine infusion caused no change in the median survival of the nude mice, which died several months after the conclusion of treatment. At most, these doses of thymidine caused transient weight loss in some of the treated animals. The biggest drop in weight occurred during the first infusion cycle, due principally to the apparent stress of the infusion procedure.
itself. When the mice were detached from the infusion apparatus during rest periods, almost all of the weight lost initially was regained. With doses of thymidine higher than 120 g/kg/cycle, increased mortality was associated with an elevation in the serum thymidine levels (Chart 2). The data reported here strongly indicate that therapeutically effective doses of the concentrated nucleoside can be infused repeatedly in mice without causing permanent toxicity or significant mortality.

Rapid necrosis of a large tumor burden, as much as 3 g or more in some cases, was the most probable cause of death for mice which responded to thymidine treatment. It should be emphasized that the doses of thymidine infused in these cycles were well within the tolerable dose range for mice without tumors and for mice bearing tumors which did not necrotize during or immediately subsequent to a thymidine cycle. Deaths occurred under similar circumstances when thymidine at 28.5 g/liter was used (18). Although no attempt was made in the present studies to prevent death from massive tumor necrosis, it was observed that host survival improved if treatment was started within 2 weeks after tumor inoculation such that the tumor mass was smaller when the response occurred.

To our knowledge, the effect of massive tumor necrosis of human xenografts on host nude mice has not been studied extensively, possibly due to the paucity of agents which cause such responses. In cancer patients, tumor necrosis can cause release of toxic substances which may result in severe uric acid nephropathy (7), pyrexia and a semicomatose state, bone necrosis which can lead to fatal hypercalcemia (20), acute psychosis (23), and possibly severe dysfunction of the immune system and deterioration of pulmonary function (20).

In earlier experiments in which thymidine treatment was started soon after inoculation of melanoma cells, there was a prolonged delay in tumor appearance and marked inhibition of subsequent tumor growth in treated compared to control mice (17). In the present experiments, which are more analogous to most clinical situations, drug treatment was initiated only after the cancer cells had formed palpable growing tumors. Under these circumstances, growth inhibition of the CA 1 melanoma was obtained after administration of 3 thymidine treatment cycles, and at least 4 cycles were required to obtain tumor destruction. With 5 cycles, 7 of 12 (58%) tumors responded, completely. Two additional animals may have responded and died from massive tumor necrosis caused by thymidine. When a critical point in thymidine treatment was reached, the entire tumor became necrotic usually within 8 hours. These results are striking in view of the refractoriness to chemotherapy of human melanomas in patients or heterotransplanted in nude mice (10). Had we restricted treatment to the protocol of Howell et al. (12), i.e., only 3 cycles at doses of 72 g thymidine/kg/cycle, our results probably would have been similar to those found by these investigators for a human melanoma heterotransplant in nude mice, e.g., partial inhibition of tumor growth but no responses.

It is not well understood why several cycles of thymidine infusion are required to cause most thymidine-sensitive human tumor xenografts to respond (18). In vitro, CA 1 cells are highly sensitive to this nucleoside; less than 1% of the cells remain viable, as determined by colony formation assays, after 72 hr exposure to 1 mm thymidine (19). The plasma levels of thymidine in our experiments were at least this high. It is possible that deoxycytidine (5, 26) or other nucleosides which counteract the cytotoxic effect of thymidine may be present at sufficient levels to delay tumor response. An alternative explanation is that increases in cellular volume (16, 21) rather than cell division may cause the major part of apparent tumor growth during treatment of thymidine-sensitive tumors and that tumor volume increases may not reflect changes in the population of viable cells (22). Another possibility is that poor tumor vascularization obstructs a significant amount of the plasma thymidine from reaching most of the viable tumor.

A steep dose response for thymidine has been found in vitro for certain human melanoma cells. Clinical reports strongly suggest that plasma concentrations of about 5 mw thymidine would be tolerated. Use of more concentrated thymidine solutions may offer practical and more convenient ways of treating patients with thymidine because higher doses could be infused with less fluid. With this approach, optimal thymidine doses can be administered for use as a single agent or in combination with other drugs (6, 19, 27).

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REFERENCES


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