

# Effect of Thymidine on the Survival of Mice with EL4 Tumors<sup>1</sup>

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## ABSTRACT

The effect of thymidine (dThd) on the growth of EL4 thymoma tumors in syngeneic C57BL/6 mice was determined. *In vitro*, EL4 cells are approximately 10-fold more sensitive to dThd than are many other tumor lines, and they are 100-fold more sensitive than are normal cells; dThd (2.5 to 5  $\mu\text{g}/\text{ml}$ ) were cytostatic, and dThd (12.5 to 25  $\mu\text{g}/\text{ml}$ ) cytotoxic to EL4 cells. The half-life of dThd in mouse serum was approximately 60 min, so that 20-g mice given injections with 24 mg dThd at 4-hr intervals during the day and with 30 mg dThd before an 8-hr interval overnight were estimated to maintain systemic concentrations of dThd greater than 25  $\mu\text{g}/\text{g}$ . Groups of 10 mice were given  $2.2 \times 10^7$  or  $2.4 \times 10^4$  EL4 cells i.p. and then treated with 0.86% NaCl or dThd on the 4- and 8-hr regimen. The mean death times of the NaCl- and dThd-treated mice were 9.9 or 16.4 days and 17.7 or 23.1 days, respectively. The 7-day differences between the death times of NaCl- and dThd-treated mice were significant to  $p < 0.0001$ , and the 7-day delay in death time was equivalent to that expected from a 3-log reduction in viable EL4 cells. The mean death time of 10 NaCl-treated mice given  $2 \times 10^2$  cells was 23.9 days, and the death time for 7 of 10 dThd-treated mice was 31.4 days. Three dThd-treated mice given  $2 \times 10^2$  cells survived until sacrificed at 60 days. The data show that although some sensitive cells may escape inhibition and replicate under the injection regimen used, dThd is cytotoxic to sensitive EL4 cells *in vivo*. The data suggest that dThd might be a useful chemotherapeutic agent for tumors that have the same level of sensitivity as EL4.

## INTRODUCTION

dThd<sup>2</sup> arrests cells at the G<sub>1</sub>-S stage of their growth cycle by inhibiting the deoxyribonucleotide reductase conversion of CDP to dCDP (8). Generally, more than  $10^{-3}$  M dThd is required to arrest normal cells (1, 10), but a significant number of tumor cell lines are arrested or killed by dThd concentrations as low as  $10^{-5}$  M (3, 4, 7, 9, 10). The apparent selective effect of dThd on tumor cells *in vitro* suggests that dThd might be useful as a chemotherapeutic agent in the treatment of dThd-sensitive tumors. This idea was tested by Lee *et al.* (5, 6) who found that dThd inhibited the growth of human melanomas in nude (athymic) mice. We have tested the effect of dThd on the growth of very sensitive EL4 thymoma cells in syngeneic C57BL/6 mice. We report here that dThd significantly prolonged the lives of EL4 tumor-bearing mice and that when the number of cells injected into the mice was reduced to minimize the presence of dThd-resistant cells in the inocula, dThd appeared to "cure" 3 of 10 tumors.

## MATERIALS AND METHODS

The EL4 cells used in these experiments were originally obtained from Dr. Frank Fitch, University of Chicago. They were maintained as ascites tumors in female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) or by *in vitro* culture in Roswell Park Memorial Institute medium 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (300  $\mu\text{g}/\text{ml}$ ), penicillin (50  $\mu\text{g}/\text{ml}$ ), streptomycin (50  $\mu\text{g}/\text{ml}$ ), and Fungizone (0.5  $\mu\text{g}/\text{ml}$ ) (Grand Island Biological Co., Grand Island, N. Y.). Viable cells were counted under the microscope as cells that excluded trypan blue dye. dThd was purchased from Sigma Chemical Co. (St. Louis, Mo.).

**Assay for dThd.** Ninety-to 200- $\mu\text{l}$  samples of blood were collected from mice by retroorbital puncture with 50- $\mu\text{l}$  heparin-coated micropipets. The samples were centrifuged at  $8000 \times g$  to remove RBC, and the supernatant sera were collected, diluted  $\frac{1}{3}$  or  $\frac{1}{8}$  with medium containing  $1 \times 10^4$  EL4 cells, and incubated in 6% CO<sub>2</sub> at 37° for 72 hr as described in Table 1. A sample of  $1 \times 10^4$  cells was fixed with 0.05% glutaraldehyde to serve as a reference to judge whether cell division had occurred during the 72-hr incubation period or whether cell division was arrested by dThd.

**dThd Injection Regimen.** Mice were given i.p. injections of 0.3-ml volumes 0.86% NaCl solution or of designated amounts of dThd dissolved in 0.86% NaCl solution. Injections of 24 mg of dThd at 8 a.m., 12 p.m., 4 p.m., and 8 p.m. and of 30 mg of dThd at 12 a.m. are referred to as a 24- and 30-mg, 4-hr day-8-hr night regimen. Injections of 24 mg of dThd at 6 a.m., 9:30 a.m., 1 p.m., 4:30 p.m., and 8 p.m. and of 30 mg at 12 a.m. are for convenience referred to as a 24- and 30-mg, 3.5-hr day-6-hr night regimen.

## RESULTS

We first determined the effects of different concentrations of dThd on the growth and viability of EL4 cells *in vitro*. Chart 1 shows that under the conditions of the assay dThd (1.25  $\mu\text{g}/\text{ml}$ ) had no measurable effect on cell replication, whereas dThd (2.0  $\mu\text{g}/\text{ml}$ ) arrested the increase in cell numbers for approximately 4 days, dThd (5  $\mu\text{g}/\text{ml}$ ) reduced the number of viable cells by 80 to 90% before the number of cells increased, and dThd (12.5 or 25  $\mu\text{g}/\text{ml}$ ) reduced the number of viable cells more than 2 logs in less than 12 days. The data suggested that *in vivo* the equivalent of dThd (12 to 25  $\mu\text{g}/\text{ml}$ ) might be adequate to cause a regression of EL4 tumors.

To estimate how much and how often dThd had to be injected into the mice to maintain a concentration equivalent to or above 12 to 25  $\mu\text{g}/\text{ml}$ , we gave 20-g mice a single i.p. injection of 2 mg dThd; at subsequent times, we assayed the concentration of dThd in the sera of the mice. Serum dThd was detected by its inhibitory effect on EL4 cell growth. A later experiment showed that the rate of clearance of dThd from the serum was the same after prolonged administration as after the single

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<sup>2</sup> The abbreviation used is: dThd, thymidine.

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injection. The data in Table 1 show that the sera of mice given injections of 0.86% NaCl solution did not inhibit the growth of EL4 cells. The addition of dThd (6  $\mu\text{g}/\text{ml}$ ) to the sera caused the complete arrest of cell replication measured at 72 hr, but 4  $\mu\text{g}$  dThd had no visible effect. The data also show that EL4 replication was inhibited by sera taken from dThd-injected mice 150 min after injection but that sera taken at 180 min had no inhibitory effect. We concluded that the serum concentration of dThd fell below 6  $\mu\text{g}/\text{ml}$  between 150 and 180 min after injection. To estimate the half-life of dThd in the serum, we assumed that the 2 mg of injected dThd were distributed rapidly and uniformly through the mice and thus that the initial concentration of dThd in the serum was 100  $\mu\text{g}/\text{ml}$ . We then calculated from a semilog plot of the data showing the decrease of dThd from 100  $\mu\text{g}/\text{ml}$  at zero time to 6  $\mu\text{g}/\text{ml}$  between 140 and 180 min that the half-life of dThd was approximately 60 min. This indicated that by injecting 15 mg dThd into 20-g mice every 3 to 4 hr, we could maintain a serum concentration of dThd that did not fall below 25  $\mu\text{g}/\text{ml}$ .

**dThd Treatment of Mice Given  $2 \times 10^7$  EL4 Cells.** We injected  $2.4 \times 10^7$  EL4 cells i.p. into 20 mice. Starting 1 hr later, 10 mice were given injections i.p. with 15 mg dThd in 0.3 ml 0.86% NaCl solution at 3- to 4-hr intervals during the day and after an 8- to 10-hr interval overnight. Ten mice, the controls, were given injections on the same schedule with 0.3 ml 0.86% NaCl solution only. Injections continued until the mice died. Chart 2, *Line C 1*, shows that the 10 control mice died in 9 to 12 days (mean, 10.4 days), whereas the dThd-treated mice died in 12 to 18 days (mean, 16 days). Thus, dThd significantly prolonged the lives of the tumor-bearing mice ( $p < 0.0001$ ). Chart 2 also shows the results of a second experiment in which 30 mice were given  $2.2 \times 10^7$  EL4 cells i.p. and then divided into 3 groups. The first group, a control, was given injections with 0.86% NaCl solution on a 4-hr day-8-hr night regimen (see "Materials and Methods"). The second

Table 1

## Assay of serum dThd

Control sera were collected from 2 mice (A and B) about 2 hr before the mice were given injections of dThd. The mice were given i.p. injections of 2 mg of dThd in 0.5 ml 0.86% NaCl solution at zero time. The mice were bled at the times indicated. Twenty or 10  $\mu\text{l}$  of serum were added to 40 or 50  $\mu\text{l}$  of medium containing  $1 \times 10^4$  EL4 cells ( $1/2$  and  $1/4$  dilutions, respectively). Each control serum sample was divided into 3 aliquots before dilution. One aliquot (control) was diluted directly  $1/2$  and  $1/4$  with cells in medium. Six  $\mu\text{g}$  dThd in 2  $\mu\text{l}$  of 0.86% NaCl solution were added to a second aliquot (control plus 6  $\mu\text{g}$  of dThd) before it was diluted  $1/2$  and  $1/4$  with cells in medium, and 4  $\mu\text{g}$  of dThd in 2  $\mu\text{l}$  were added to the third aliquot (control plus 4  $\mu\text{g}$  of dThd) before it was diluted  $1/2$  and  $1/4$  with cells in medium. The EL4 cells were incubated with the different serum samples in flat-bottom wells of a microtiter plate for 72 hr at 37° in 6%  $\text{CO}_2$ .

Serum	$1/2$ dilution		$1/4$ dilution	
	A	B	A	B
Control	— <sup>a</sup>	—	—	—
Control plus 6 $\mu\text{g}$ dThd	+	+	—	—
Control plus 4 $\mu\text{g}$ dThd	—	—	—	—
dThd (min)				
30	+	+	+	+
60	+	+	+	+
90	+	+	+	+
120	+	+	—	—
150	+	+	—	—
180	—	—	—	—
240	—	—	—	—

<sup>a</sup> —, dThd wells containing at least four times the number of cells that were present at the start of the incubation period (see "Materials and Methods"); +, dThd wells in which no increase in cell number was detected.

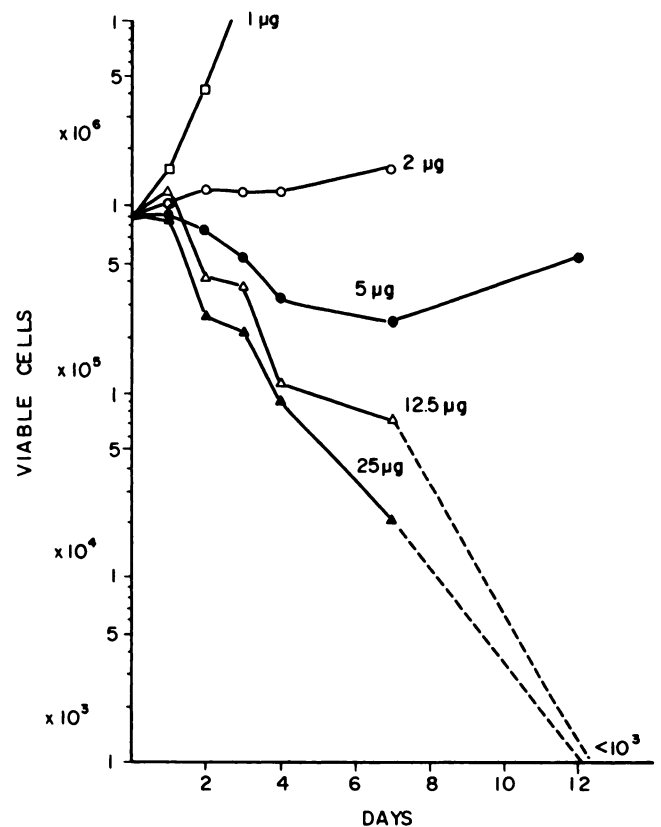


Chart 1. Viability of EL4 cells grown with different concentrations of dThd. Approximately  $9 \times 10^5$  cells in 5 ml of medium containing the indicated concentrations of dThd were incubated in graduated culture tubes at 37° and 6%  $\text{CO}_2$ . At the times shown, the number of viable cells in 4 samples of each culture was determined by counting cells that excluded 0.04% trypan blue dye. On the fourth day of incubation, cells that were grown with dThd (5, 12.5, or 25  $\mu\text{g}/\text{ml}$ ) were concentrated 5-fold by removing all but 1 ml of medium. At 12 days, no viable cells were detected in any of 10 samples of the (12.5- or 25- $\mu\text{g}/\text{ml}$ ) dThd cultures. Since each microscope field surveyed would have shown 1 cell for every  $1 \times 10^3$  cells in culture, the 12-day samples were recorded as having less than  $10^3$  viable cells.

group was given injections with 24 and 30 mg dThd on the same 4-hr day-8-hr night regimen, and the third group was given 24 and 30 mg dThd on a 3.5-hr day-6-hr night regimen. We had found that 20-g mice could tolerate up to 32 mg dThd on the 4-hr regimen, but that they died in about 12 days if given 40 mg. The mean death time of the control mice was 9.9 days (Chart 2, *Line C 2a*), and the mean death times of the 2 treated groups were 17.4 and 17.7 days (*Lines dThd 2b* and *dThd 2c*). The differences between the mean death times of the control and the treated mice were significant ( $p < 0.0001$ ). Autopsies of the control and treated mice showed that the peritoneal cavities of the control mice were filled with a creamy suspension of large cells, presumably EL4 cells, at a concentration of about  $1 \times 10^8$  cells/ml. The spleens of the control mice were enlarged and filled with large EL4 or blast-like cells. In contrast, the dThd-treated mice had very few cells in their peritoneal cavities, and they had normal-looking spleens. The spleens, however, contained a number of large EL4 or blast cells. Neural metastases of EL4 cells are supposedly the proximal cause of death, and since the dThd-treated mice resembled the control mice in that both were lethargic and moved stiffly just before death, we presume that both groups of mice died of neural metastases. In any case, dThd clearly prolonged the lives of

the mice that were given a massive burden of tumor cells. The dThd decreased the effective tumor burden from  $2 \times 10^7$  cells which caused death in 10 days to the equivalent of about  $2 \times 10^4$  cells which caused death in approximately 16 days (see below).

**dThd Treatment of Mice Given  $2.4 \times 10^4$  or  $2 \times 10^2$  EL4 Cells.** In a second series of experiments, mice were given  $2.4 \times 10^4$  cells i.p. and then treated until the time of death with 0.86% NaCl solution or 24 and 30 mg dThd on the 4-hr day-8-hr night regimen. Chart 3 shows that the mean death time of 10 mice that were treated with NaCl solutions was 16.4 days and that the mean death time of 10 mice that were treated with dThd was 23 days ( $p < 0.0001$ ). The dThd-treated mice were autopsied and found to resemble the mice that were given  $2 \times 10^7$  cells except that the peritoneal cavities contained at least  $10^6$  recoverable EL4-like cells. In this experiment as in the first, dThd treatment prolonged the lives of the mice by approximately 7 days. dThd could have this effect if it acted as an imperfect cytostatic agent that slowed the growth of the tumor cells without killing them or if it acted as a cytotoxic agent that reduced the number of viable cells approximately 1000-fold.

If dThd reduced the number of viable cells by 3 logs, mice that were given  $2 \times 10^2$  cells and then treated with dThd should survive the tumors. We tested this idea by injecting  $2 \times 10^2$  cells i.p. into mice and then treating the mice with 24

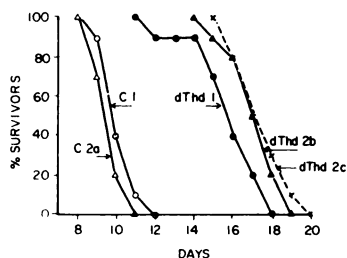


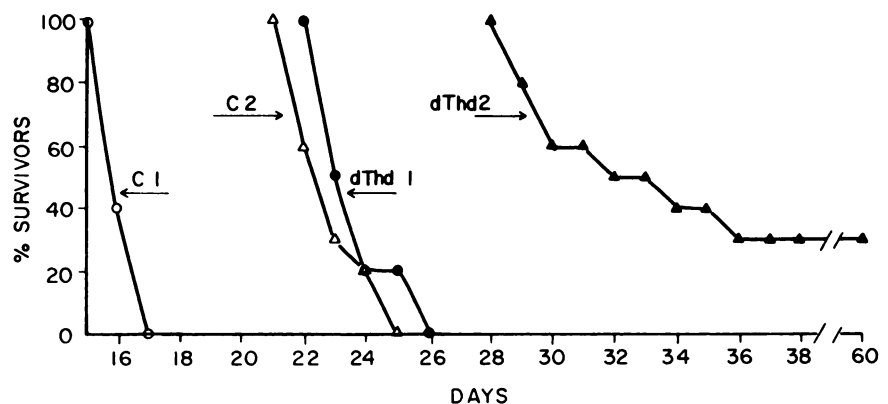
Chart 2. The effect of dThd on the survival of C57BL/6 mice with EL4 tumors. The results of 2 experiments are shown. In the first experiment, 2 groups of 10 mice were given injections i.p. with  $2.4 \times 10^7$  EL4 cells. One group, the control (C 1), was given injections i.p. with 0.86% NaCl solution at 4-hr intervals during the day and after an 8- to 10-hr interval overnight. The second group (dThd 1) was given injections i.p. with 15 mg of dThd on the same schedule. In the second experiment, 3 groups of 10 mice were given injections i.p. with  $2.2 \times 10^7$  EL4 cells. The control group (C 2a) was given 0.86% NaCl solution on a 4-hr day-8-hr night regimen (see "Materials and Methods"). A second group (dThd 2b) was given injections i.p. with 24 and 30 mg of dThd on a 4-hr day-8-hr night regimen. The third group (dThd 2c) was identical to the dThd 2b group except that these mice were given injections on a 3.5-hr day-6-hr night regimen.

and 30 mg dThd on the 4-hr day-8-hr night regimen. In this experiment, the 10 control mice (Chart 3, Line C 2) died in 22 to 25 days (mean, 23.9 days), and 7 of the dThd-treated mice (Line dThd 2) died between 28 and 36 days (mean, 31.4 days). dThd treatment of the 3 surviving mice was stopped after the 40th day, and the mice were sacrificed for autopsy on the 60th day. A microscopic examination of the spleens from the 3 survivors showed no abnormalities, and no cells were recovered after long-term culture of the disrupted spleens or of cells washed from the peritoneal cavities.

**Frequency of dThd-resistant Cells in EL4 Cultures.** The deaths of the dThd-treated mice could be explained if there were dThd-resistant EL4 cells among the cells injected into the mice or if resistant cells arose during the treatment of the mice. Two experiments were done to determine the likelihood of these events. In one, we tested the dThd sensitivity of a culture of EL4 cells that had been cultured *in vitro* for nine months. We incubated  $2 \times 10^7$  cells in 40 ml of medium containing dThd ( $17 \mu\text{g/ml}$ ). Chart 4 shows that the number of viable cells in the culture decreased approximately 2 logs in 8 days and that after remaining relatively constant for approximately 3 days, the number of viable cells increased, doubling in about 48 hr. The surviving cells were able to replicate when subcultured in dThd ( $50 \mu\text{g/ml}$ ). The data do not indicate whether a portion of the original cell population was able to adapt to growth in dThd or a fraction of the original population was constitutively resistant to dThd. If we assume the latter, we can calculate that the original population of  $2 \times 10^7$  cells contained approximately  $6 \times 10^3$  dThd-resistant cells that replicated every 48 hr to produce the  $3.5 \times 10^5$  dThd-resistant cells found in the culture at 11 days.

Our second test for dThd-resistant EL4 cells was done on cells taken at autopsy from the peritoneum of one of the mice that were given  $2.4 \times 10^4$  EL4 cells and then treated with dThd. The cells were diluted to a concentration of  $1.5 \times 10^5$  / ml in culture medium and then incubated in the presence or absence of dThd ( $25 \mu\text{g/ml}$ ). Chart 4 shows that in the absence of dThd, the number of viable cells decreased slightly to  $1.2 \times 10^5$  cells/ml and then increased slowly, doubling in approximately 48 hr. In the presence of dThd, the number of viable cells in the culture decreased 90% and then increased with a doubling time of approximately 48 hr. We calculate by extrapolating the 48-hr growth curve that at the start of incubation, approximately 0.3% of the cells taken from the peritoneum were dThd resistant.

Chart 3. The effect of dThd on the survival of mice given  $2.4 \times 10^4$  or  $2 \times 10^2$  EL4 cells. The results of 2 experiments are shown. In the first experiment, 20 mice were given  $2.4 \times 10^4$  EL4 cells i.p. Ten control mice (C 1) were given injections i.p. with 0.86% NaCl solution, and the other 10 mice (dThd 1) were given injections with 24 and 30 mg of dThd on a 4-hr day-8-hr night regimen. In the second experiment, 20 mice were given  $2 \times 10^2$  EL4 cells i.p. Ten control mice (C 2) were treated with 0.86% NaCl solution, and 10 mice (dThd 2) were treated with 24 and 30 mg of dThd on the 4-hr day-8-hr night regimen as before.



## DISCUSSION

The data show clearly that dThd increased the mean death times of mice given injections of  $10^2$  to  $10^7$  tumor cells approximately 7 days beyond the mean death times of equivalent untreated mice. This 7-day difference was statistically significant ( $p < 0.0001$ ). If dThd were known unequivocally to be cytotoxic to EL4 cells *in vivo*, we could ascribe the 7-day delays entirely to a 3-log reduction in the number of viable tumor cells in the mice. The survival of 3 of 10 mice that were given 200 EL4 cells is evidence for dThd cytotoxicity *in vivo*. However, the deaths of the 7 mice suggest that dThd did not reduce the number of viable cells by a full 3 logs. The 7 mice might have died because there were dThd-resistant cells among the 200-cell inocula. If this were so, a Poisson calculation

$$p(r) = \left(\frac{n^n}{r!}\right) e^{-n}$$

where  $p(r)$  is the probability of  $r$  dThd-resistant cells being present in a given sample when the average number of dThd-resistant cells/sample is  $n$  indicates that for 30% of the mice to have received zero resistant cells, the average number of dThd-resistant cells in each inoculum was 1.2. Thus, the frequency of dThd-resistant cells would have been about 0.6%. This high frequency is not ruled out, but it is higher than expected from the estimated 0.3% frequency of resistant cells taken from the dThd-treated mouse, *i.e.*, after growth under enrichment conditions.

The dThd-resistant cells from the treated mouse and from the *in vitro* culture were challenged only with dThd (50  $\mu\text{g/ml}$ ). Thus, the exact level of their resistance is unknown. The cells may have mutated from their original sensitivity to approximately  $2 \times 10^{-5}$  M, to the sensitivity of normal cells, or to the intermediate sensitivity of many tumor cell lines (1, 7, 9, 10). The fact that cells may have different levels of sensitivity to dThd suggests that there may be multiple factors that determine sensitivity of resistance. If this were so, there should be more than one way to mutate to resistance, the frequency and variety of resistant mutants should be increased, and the effects of dThd treatment of tumors would be highly variable.

It is most probable that one or more of the dThd-sensitive cells in the 200-cell inocula escaped the effects of dThd either by lodging in privileged sites where the concentration of dThd was not constantly above the minimum level needed to arrest the cells or because the cells were at sites where local conditions, *e.g.*, the availability of deoxycytidine mitigated the effects of dThd. The recovery of more than  $10^6$  cells from a dThd-treated mouse that was given initially only  $2.4 \times 10^4$  cells shows that some replication of dThd-sensitive cells may occur in treated mice. It might be possible to reduce the number of cells that escape dThd inhibition and thus increase the number of surviving mice by improving the schedule and route of dThd injections.

We chose the EL4 cell-C57BL/6 mouse system to test the effects of dThd or tumor growth for 2 reasons. One is that the EL4 cells are among the most sensitive to dThd *in vitro* (7, 10), and they should therefore produce clear-cut responses to dThd *in vivo*. The second reason is that the syngeneic C57BL/6 mice do not mount an obvious immune response that would

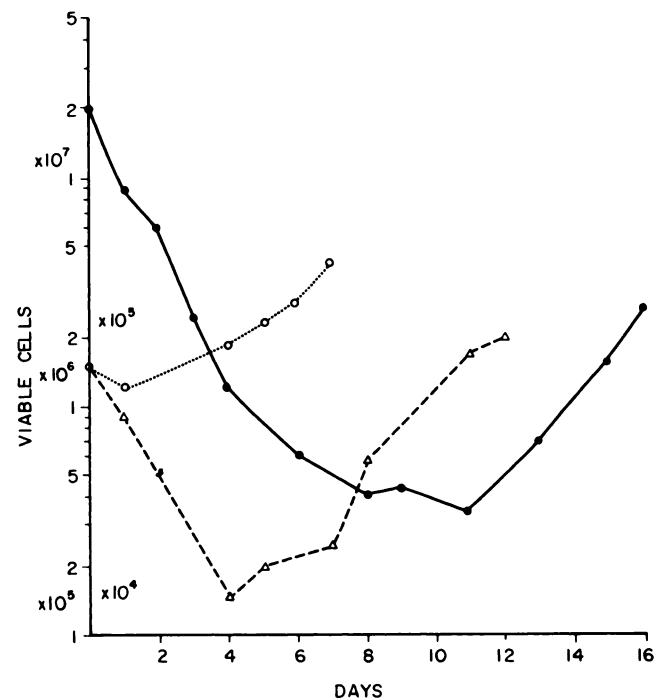


Chart 4. The presence of dThd-resistant cells in EL4 populations. The results of 2 experiments are shown. In the first experiment, approximately  $2 \times 10^7$  EL4 cells from a culture grown *in vitro* were incubated with dThd (17  $\mu\text{g/ml}$ ) (●). At the times shown, the numbers of viable cells in the culture were assayed as described in Chart 1. Scale outside graph, the numbers of viable cells. In the second experiment (*inner scale*), cells were taken at autopsy from one of the mice (Chart 3, *dThd 1*) that had been given  $2.4 \times 10^4$  EL4 cells and treated with dThd. The cells were divided into 2 cultures of  $1.5 \times 10^5$  cells/ml and incubated with dThd (25  $\mu\text{g/ml}$ ;  $\Delta$ ) or without dThd (○).

materially distort the EL4 response to dThd (2). The choice of the EL4-C57BL/6 system was validated by an experiment<sup>3</sup> that tested the effects of dThd on plasmacytoma 4T00.1 cells that were approximately 10-fold less sensitive to dThd than were EL4 cells but 10-fold more sensitive than were normal cells. That is, *in vitro*, the 4T00.1 cells were not affected by dThd (5  $\mu\text{g/ml}$ ), but they were arrested by dThd (25  $\mu\text{g/ml}$ ). The experiment in which syngeneic BALB/c mice were given  $2 \times 10^6$  4T00.1 cells and then treated with 15 and 24 mg dThd on the 4-hr day-8-hr night injection regimen showed that the mean death times of the treated mice were not significantly different from those of control mice. It is probable that the systemic concentrations of dThd were not high enough consistently to affect the growth of a significant number of 4T00.1 cells.

Taken together, the data suggest that dThd might be a clinically useful chemotherapeutic agent for tumors but that best results could come from its use on tumors that resembled EL4 tumors in their sensitivity to dThd.

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