Effects of Thymidine and Thymidine Plus 5-Fluorouracil on the Growth Kinetics of a Human Lymphoid Cell Line

Anthony J. Muro, Jerrold Fried, David Burchenal, Karen L. Vale, Annabel Strife, Thomas Woodcock, Charles W. Young, and Bayard D. Clarkson

ABSTRACT

We have studied the effects of thymidine (dThd) alone and in combination with 5-fluorouracil (FUra) on the survival and growth kinetics of a rapidly growing lymphoid cell line, SK-L7, in suspension culture. Continuous exposure to $10^{-2}$ M dThd increased the doubling time from 14 hr in the untreated cultures to 24 hr and reduced the cloning efficiency by about 50% after 72 hr exposure. At a $10^{-2}$ M concentration, dThd caused a progressive decline in trypan blue-excluding cells and a 98% reduction in the cloning efficiency by 72 hr, but failed to kill all the cells even after 6 days. Flow cytometric measurements of propidium iodide-stained cells showed a maximum accumulation of cells in S phase after 12 hr exposure, 74 and 85% with $10^{-3}$ and $10^{-2}$ M dThd, respectively, as compared to 61% in the cells even after 6 days. Flow cytometric measurements of the media by promoting the conversion of FUra to 5-fluoro-2'-deoxyuridine; HPLC, high-pressure liquid chromatography.

INTRODUCTION

dThd in concentrations exceeding $10^{-4}$ M inhibits DNA synthesis (9, 22) and causes a reversible block in the S phase of the cell cycle of various cell lines (22, 25, 30). Irreversible toxicity has been reported when the cells were exposed to $10^{-3}$ M dThd for longer than one generation (11). There has been recent interest in the antitumor effect of dThd alone (14, 15, 27) and in combination with other drugs (10, 20, 21, 23, 24, 29). It has been reported that tumor-producing cell lines are more sensitive to the lethal effects of dThd than are their non-tumor-producing counterparts (14). Continuous infusion of high concentrations of dThd into nude mice has been reported to inhibit the growth of transplanted human melanoma cells and prolong survival, with no apparent toxicity (15). dThd potentiates the antitumor activity of FUra in vivo in mice (16, 20, 23) and increases the biological effects of FUra in humans (29).

Because of the potential therapeutic value of dThd singly or in combination with other agents, we undertook an extensive investigation of the cytotoxic and cytokinetic effects of dThd alone and in combination with FUra on the growth kinetics and survival of a human lymphoid cell line whose cytokinetic properties have been well defined.

MATERIALS AND METHODS

Cell Line. The cell line used, SK-L7, was derived from the peripheral blood of a child with acute leukemia (1). The cells probably originated from normal B-lymphocytes which acquired their capacity for unlimited proliferation as a result of transformation by Epstein-Barr virus (2). In addition, the fast-growing subline of SK-L7 used in the experiments reported herein have trisomy of chromosome 15 (2). During exponential growth, these cells have an average doubling time of about 14 hr, a pulse $[^3H]$dThd labeling index of about 60%, and a cloning efficiency of about 60%. The cells were maintained in suspension culture in McCoy's Medium 5A (modified), supplemented with 20% fetal calf serum at 37°C in an atmosphere of 5% CO2. Drugs were added to exponentially growing cultures with an initial cell density of about $1 \times 10^6$ cells/ml and a final volume of 50 ml in Falcon T-75 flasks. Cultures were diluted with fresh media or fresh media plus an appropriate concentration of drug every 2 to 3 days to keep the cell density from exceeding $6 \times 10^6$ cells/ml. Cell numbers were determined by sample counts on a hemocytometer and viability by trypan blue exclusion.

Drugs. dThd (obtained from the Division of Cancer Treatment, National Cancer Institute, NIH), thymine (Calbiochem, San Diego, Calif.), and FUra (Roche Laboratories, Nutley, N. J.) were diluted in McCoy's medium. Five ml volumes of these stocks were added to the cultures to obtain the desired final concentration. The same compounds were used as chromatographic standards. FdUrd was obtained from Roche Laboratories, and $[2-14C]$FUra (specific activity, 60 mCi/mm) was from Amershamp Corp., Arlington Heights, Ill.

Chemical Analysis. Chromatographic separation and quantitation of dThd, thymine, FUra, and FdUrd were done using reverse-phase HPLC as described previously (13, 28). Since FUra and FdUrd concentrations were near or below the limits of reliable quantitation by UV absorption detection, we used $[^3H]$FUra to increase sensitivity for these materials. Samples to be analyzed for FUra and FdUrd had approximately $10^{-5}$ M...
concentrations of unlabeled FUrA and FdUrd added just prior to analysis to aid the definition of exact retention times. FUrA and FdUrd peaks identified by UV absorption were hand collected, and the $^{14}$C was quantitated by liquid scintillation counting, using a Packard Model 3380 quench-correcting instrument with a Model 544 Quench Correcting System (Packard Instruments, Downers Grove, Ill.).

Overall sensitivity of the analytic methods was approximately 2 $\mu$m for dThd and thymine and 50 nM for FUrA and FdUrd using $^{14}$C-labeled materials.

**Cloning Efficiency.** Cloning efficiencies of control and drug-treated cultures were determined by calculating the percentage of cells forming colonies in semisolid media (2). A bottom layer of 1 ml of 0.5% agar with 50% fetal calf serum in McCoy's Medium 5A (modified) without cells was placed in Petri dishes (10 x 35 mm). After hardening, this was overlaid with a top layer containing the cells (washed to remove drug) in 1 ml of 1% methyl cellulose with 20% fetal calf serum in the same medium. Usually, 100 untreated and 100 to 300 drug-treated “viable” (trypan blue-excluding) cells were plated, depending upon the anticipated cloning efficiency. It had previously been determined that the presence of dead cells has no influence on the cloning efficiency of the remaining live cells (2). Ten to 16 Petri dishes were plated for each group tested, and colony counts were done after 7 days of incubation at 37° in an atmosphere of 5% CO₂. Although their size varied, most colonies contained many hundreds of cells by this time; colonies with less than 40 cells were rare and were not counted.

**Mitotic Indices.** Smears were prepared by cytocentrifugation of culture samples and stained with tetrachrome. The mitotic index was determined by calculating the percentage of 500 to 1000 cells counted that were in mitoses.

**Cell Cycle Distribution Analysis.** The technique of cytofluorometric analysis of cell cycle distribution of propidium iodide-stained cells has been described in detail elsewhere (7). Briefly, the relative DNA content of approximately $10^5$ cells was determined after staining with propidium iodide. Cell fluorescence was measured with a Model 4802 Cytofluorograf (BioPhysics Systems, Inc., Mahopac, N. Y.), and the data were collected using a Northern Model NS-602 multichannel pulse height analyzer (Tracor-Northern Scientific, Inc., Middleton, Wis.), recorded on a Model ASR 33 Teletype printer and paper tape punch, and analyzed using a PDP 11/70 computer. The method of quantitative analysis to estimate the distribution of cells among the phases of the mitotic cycle has been described elsewhere (6).

**RESULTS**

**Effect of dThd on the Growth and Survival of SK-L7.** Chart 1 shows the effect of various concentrations of dThd on the growth of SK-L7 in suspension culture. Continuous exposure to $10^{-4}$ M dThd had no effect on growth rate, but higher concentrations were inhibitory. dThd ($10^{-3}$ M) prolonged the doubling time to about 24 hr, and $10^{-2}$ M dThd caused a progressive decline in the number of viable cells recovered. Viabilities, as determined by trypan blue exclusion, were 95, 94, 92, and 86% and 88, 46, 35, and 9% after 1, 2, 3, and 6 days of exposure to $10^{-3}$ and $10^{-2}$ M dThd, respectively, as compared to 97% in the untreated cultures.

Following treatment with dThd in suspension culture, the cloning efficiency of the recovered viable cells was determined (Chart 2). dThd ($10^{-3}$ M) reduced the cloning efficiency by 50% at 72 hr, but longer exposure was no more effective. dThd ($10^{-2}$ M) caused a 98% reduction in cloning efficiency at 72 hr but failed to kill all the cells even after 6 days of exposure. If one considers the whole population, the reduction in cloning efficiency is of course greater than the above-stated values because of cell death; however, the precise values cannot be calculated, because cell death cannot be accurately quantitated in this system.

**Effect of dThd on the Cell Cycle of SK-L7.** dThd in concentrations of $10^{-3}$ M and greater caused marked perturbations of the cell cycle; $10^{-4}$ M dThd had no measurable effect. Chart 3
showed the DNA cytofluorograms, mitotic indices, and calculated fractional distributions of cells in G1, S, and G2 + M compartments of cultures treated with 10^{-2} and 10^{-3} M dThd after various times of exposure. After 3 to 6 hr (not shown), there was a depletion of cells in G2 + M and an accumulation of cells in S phase. This accumulation was maximal after 12 hr exposure to 10^{-3} and 10^{-2} M dThd, with 74 and 85% in S phase, respectively, as compared to 61% in the untreated cells. During the period of S-phase accumulation, the G1 compartment became markedly depleted. By 72 hr, the cell cycle phase distributions of the treated cells at 10^{-3} M gradually returned almost to the unperturbed state; at the higher drug concentration, significant perturbation was still evident, but the distribution was gradually approaching that of the control. There was also considerable fluorescent debris to the left of the G1 peak, probably representing degenerating or dead cells.

Effect of Thymine on the Growth and Cell Cycle of SK-L7. Suspension cultures of SK-L7 were treated with 10^{-3} and 10^{-4} M thymine for up to 3 days. There was no effect on cell growth or cell cycle. The use of higher concentrations was technically precluded because of the low solubility of thymine in the media.

Effect of FUra on the Growth and Cell Cycle of SK-L7. The inhibitory effect of FUra on the growth of SK-L7 was dependent upon the dose and duration of exposure to the drug. The effect of continuous exposure of various concentrations of FUra on the growth in suspension culture is shown in Chart 4. FUra (10^{-7} M) had no effect on growth, whereas 5 \times 10^{-7} M FUra prolonged the doubling time to about 24 hr. FUra (10^{-6} M) appeared to arrest growth after 24 hr, and 10^{-5} M FUra caused a progressive decline in the number of viable cells recovered.

Effects on the cell cycle were variable and depended upon the concentration and duration of exposure to FUra. DNA cytofluorograms of FUra-treated cells are shown in Chart 5. In general, exposure to 10^{-5} M FUra resulted in an accumulation or block at the G1-S interphase or in early S phase and a depletion in the G2 + M compartment; a temporary accumulation of cells in the G2 + M compartment was noted at 48 hr, but this compartment was again depleted after 72 hr. Lower concentrations of FUra (5 \times 10^{-7} and 10^{-6} M) caused a delay in S-phase progression and a depletion of the G1 compartment.
which was most marked after 72 hr exposure. FUra (10⁻⁷ M) had no effect until 72 hr, where a moderate accumulation in S phase was noted (not shown).

Effect of dThd Plus FUra on the Growth, Survival, and Cell Cycle of SK-L7. SK-L7 cells were exposed continuously to 10⁻³ M dThd and 5 × 10⁻⁷ M FUra alone and in combination. The inhibitory effect of dThd plus FUra on growth in suspension culture was less than additive, since this combination inhibited growth to the same extent as did dThd alone (Chart 6). Cloning efficiencies after 72 hr exposure were 13.0 ± 7.9 (S.D.), 36.1 ± 12.4, and 36.2 ± 10.6 in cultures treated with FUra, dThd, and dThd plus FUra, respectively, as compared to 61.1 ± 7.4 in the untreated control; these results represent the means of 16 to 20 plates from 2 experiments. Therefore, dThd protected against, rather than potentiated, the cytotoxicity of FUra. When the drugs were administered together in these concentrations, the predominant effect on the DNA cytofluorograms (Chart 7) was that of dThd, and it appeared that dThd prevented the accumulation of cells in S phase that occurred after 72 hr exposure to FUra alone.

Other experiments examined the effects of various concentrations of FUra and dThd in combination on the growth of SK-L7. Cells were pretreated with 10⁻² or 10⁻³ M dThd for 15 min to 6 hr prior to the addition of 10⁻³ or 10⁻⁴ M FUra for another 3 to 8 hr before washing the cells and observing growth in drug-free media. In no case was the cytotoxicity of FUra plus dThd greater than that of FUra alone.

Levels of dThd, Thymine, FUra, and FdUrd in the Culture System. At intervals following the addition of 10⁻³ M dThd, the levels of dThd and thymine in the culture media were determined by HPLC. There was a slight but progressive decline in the concentration of dThd during incubation with SK-L7, from 1.4 × 10⁻³ M at 0 hr to 1.1 × 10⁻³ M at 72 hr. This slight fall in dThd was accompanied by an almost equivalent rise in the level of thymine, from less than 0.1 × 10⁻⁴ M to 1.6 × 10⁻⁴ M at 72 hr. When dThd was added and incubated in McCoy's medium containing 20% fetal calf serum in the absence of SK-L7 cells, conversion of dThd to thymine was not detected.

Cultures were treated with 5 × 10⁻⁷ M [2⁻¹⁴C]FUra with and without 10⁻³ M dThd. The concentration of FUra and FdUrd in the medium was determined by measuring the specific activity of label in the corresponding HPLC peak (Chart 8). In the cultures containing dThd, the concentration of FUra progressively fell from 5 × 10⁻⁷ M to 1.4 × 10⁻⁷ M by 72 hr; this fall in FUra was accompanied by an equivalent rise in FdUrd. This is in contrast to the very slight conversion of FUra to FdUrd which occurred in the absence of dThd. The concentration of FUra did not fall, and FdUrd did not form when FUra and dThd were added and incubated in McCoy's medium containing 20% fetal calf serum without SK-L7 cells. The conversion of dThd to thymine was not influenced by the presence of FUra or FdUrd.

DISCUSSION

In this report, we demonstrated the effects of dThd and dThd plus FUra on the survival and growth kinetics of the human lymphoid cell line SK-L7 in vitro. At concentrations of 10⁻³ M and greater, dThd inhibits growth in suspension culture and substantially reduces the cloning efficiency. The cytotoxicity of high concentrations of dThd is presumably due to a disturbance in DNA synthesis resulting from feedback inhibition by the dThd metabolite, TTP, on several steps of its synthetic pathway (3). Most important is the inhibition of CDP reductase, which results in a deprivation of dCTP and thus interferes with DNA synthesis (9). The reversal of dThd toxicity by the addition of deoxycytidine to the culture medium, which bypasses this blockade, is further evidence in support of this theory (18). The frequent application of high concentrations of dThd as a synchronizing agent is based upon its ability to cause a block in DNA synthesis which can be reversed by the removal of excess dThd or by the addition of deoxycytidine to the medium (30). Our flow cytometric analysis of SK-L7 indicates that, besides growth inhibition, dThd at concentrations of 10⁻² and 10⁻³ M causes a marked perturbation of the cell cycle. S-phase progression is prolonged, and there is a marked accumulation in S-phase after 12 hr exposure to the drug. Thus, it seems that DNA synthesis continues concurrently with growth inhibition and reduced survival.

There is considerable variability in the sensitivity of different cell lines to the toxic effects of dThd (14, 18, 26). Although 10⁻² M dThd is markedly cytotoxic to SK-L7 cells, it fails to sterilize the cultures even after 6 days of exposure. Also, after 72 hr treatment, the cell cycle phase distribution of the remaining viable cells begins to approach that of the control again. Whether the proportion of cells which survive is inherently resistant to high levels of dThd or resistance develops upon prolonged exposure through some adaptation mechanism or mutation is yet to be determined. Resistance of other cell lines to high levels of dThd has been reported (5, 18). Cells may become resistant because of a depression of dThd kinase activity or an inability to transport dThd across the cell mem-
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24 Hours

G1 = 0.27
S = 0.02
G2+M = 0.71

48 Hours

G1 = 0.27
S = 0.02
G2+M = 0.71

72 Hours

G1 = 0.28
S = 0.01
G2+M = 0.71

Chart 5. DNA cytofluorograms and cell cycle phase distributions of SK-L7 cells during continuous exposure to various concentrations of FUra (FU). Vertical lines show the positions of the observed peaks of simultaneously run untreated cultures.

Relative DNA Content

The studies with 10^{-3} \text{ M} dThd and 5 \times 10^{-7} \text{ M} FUra described above, in which dThd actually provided protection against the toxic effects of FUra, merit further comments. The minimum concentration of FUra producing detectable growth retardation is 5 \times 10^{-7} \text{ M}. In the absence of dThd, this concentration was well maintained in the medium throughout the experiment. In the presence of dThd, FUra concentrations dropped to nontoxic levels. The labeled FUra reappeared as FdUrd, which would not be growth inhibitory in the presence of dThd (17). The first step in the degradation of dThd consists of the cleavage of the glycosidic bond to form thymine (T) and deoxyribose 1-phosphate (dR-1-PO_4) by dThd phosphorlase (3):

\[
dThd + P_i \rightleftharpoons T + dR-1-PO_4
\]

This reversible reaction accounts for the small quantity of dThd present in our cultures (5). Also, after continued exposure, dThd resistance may be related to a feedback inhibition of dThd phosphorylation by TTP or to the incorporation of deoxycytidine salvaged from the DNA of dying cells (3). Perhaps the selective lethal effect of dThd on tumor cells compared to normal cells (14, 25, 26) is, in part, due to a relative incapacity of tumor cells to adapt or develop resistance to this agent. Alternately, normal cell lines may be relatively resistant to the lethal effects of dThd because they are constrained from initiating DNA synthesis. By contrast, transformed or cancer cells succumb because they proceed to synthesize DNA in a milieu deficient in dCTP. Certainly, this is an issue which deserves further study.

Several in vivo investigations have shown that dThd can potentiate the antitumor effect of FUra in mice (16, 19, 23) and in humans (29). It has been proposed (16, 19) that potentiation of the therapeutic effect of FUra occurs because dThd augments the incorporation of FUra into RNA rather than its effect on DNA synthesis. Human trials of FUra in combination with dThd (12, 29) have shown that dThd markedly prolongs the plasma half-life of FUra by inhibition of its degradation. Since the cells in our culture experiments do not catabolize FUra to any significant degree (Chart 8), they provide a system in which the effects of dThd on FUra can be assessed independently of degradation. In none of our studies did dThd increase the cytotoxicity of FUra. Also, dThd failed to significantly enhance the cytotoxicity of FUra for HeLa cells (8).
thymine which accumulates in our culture system. dThd phosphorylase, which is active in or released from SK-L7, also mediates the interconversion between FUra and FdUrd:

\[ \text{FUra} + d\text{R-1-PO}_4 \rightarrow \text{FdUrd} + P_i \]

Presumably, dThd accelerates the conversion of FUra to FdUrd, as confirmed by our culture system, by generating significant quantities of deoxyribose 1-phosphate from dThd in the formation of thymine. Our data support the concept that in vivo dThd potentiates the cytotoxicity of FUra largely by blocking the degradation of FUra (12, 29).

**Chart 6.** Change in the relative number of trypan blue-excluding cells during continuous exposure to \(10^{-3}\) M dThd (TdT) and/or \(5 \times 10^{-7}\) M FUra (FU). dThd was added 1 hr prior to FUra. Each point represents the mean of 2 experiments.

**Chart 8.** FUra (FU) levels and FdUrd (FdUrd) levels in the media of cultures initially treated with \(5 \times 10^{-7}\) M FUra with and without dThd. Control cultures contained media without SK-L7 cells.

**Chart 7.** DNA cytofluorograms and cell cycle phase distributions of SK-L7 cells during continuous exposure to \(10^{-3}\) M dThd (TdT) and/or \(5 \times 10^{-7}\) M FUra (FU). Vertical lines show the positions of the observed peaks of simultaneously run untreated cells.
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