Thymidine as a Chemotherapeutic Agent: Sensitivity of Normal Human Marrow, Peripheral Blood T Cells, and Acute Nonlymphocytic Leukemia

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Normal marrow granulocyte (CFU-GM) and peripheral blood T-lymphocyte (CFU-TL) colony-forming cells were studied for their sensitivity to high concentrations of thymidine (dThd) and compared to leukemic CFU from patients with acute nonlymphocytic leukemia (ANLL). The sensitivity of two ANLL cell lines was also assessed. dThd was toxic to both CFU-GM and CFU-TL at concentrations above 10^{-8} M when cultured under conditions where dThd exposure was analogous to that used in clinical trials. There was little variation in the fractional colony survival between marrow samples, and the sensitivity of CFU-GM closely approximated that of CFU-TL. Thymidylate was not toxic at up to 10^{-2} M. In liquid culture, T cells in G_0 at the start of exposure were able to proliferate in the presence of 10^{-3} M dThd, whereas T cells already proliferating in response to phytohemagglutinin (PHA) at the start of dThd exposure were killed. Leukemic CFU demonstrated marked variability in dThd sensitivity; blasts from some patients were resistant to dThd, while others were >100-fold more sensitive than normal CFU-GM.

HIGH CONCENTRATIONS of thymidine block DNA synthesis in proliferating cells through feedback inhibition of ribonucleotide reductase by increased intracellular concentrations of thymidine triphosphate (dTTP).^{1-4} This effect is reversible after short periods of exposure, and thymidine (dThd) has long been used to synchronize cells in culture.^{5-9} However, when exposure is maintained for long periods, dThd is also cytotoxic.^{6,10} Recently, several investigators have reported that dThd is more toxic to some types of malignant cells in culture than to normal cells from the same tissue^{11,12} and that infusion of very large doses of dThd slows the growth of some types of human tumor xenografts growing in athymic mice.^{13,14}

On the basis of these findings, dThd was introduced into clinical trial.

Preliminary clinical results^{15,16} and in vitro studies of human cell lines^{17-19} suggest that the most dThd-sensitive normal tissues of the body are likely to be the bone marrow and the T-lymphocyte population. In the present study we have measured the toxicity of dThd and its first metabolite, thymine, to progenitor cells of both of these tissues using colony-forming assays and have examined the effect of the proliferative rate of normal T cells on dThd sensitivity. The dThd sensitivity of marrow granulocyte/macrophage (CFU-GM) and T-lymphocyte (CFU-TL) progenitors was compared to that of CFU in six samples of leukemic blasts from five patients with various types of acute nonlymphocytic leukemia (ANLL) and two tissue culture lines derived from patients with ANLL. The results indicate that dThd is toxic to both normal marrow CFU-GM and T lymphocytes at concentrations above 10 \mu M. In addition, the proliferative state prior to dThd exposure is an important determinate of dThd effect. The leukemic CFU demonstrated a broad range of dThd sensitivity, but in some cases, the blasts were significantly more sensitive to dThd than normal marrow CFU-GM, suggesting that for some patients with ANLL, dThd alone or in combination with other agents^{20-22} may be a useful chemotherapeutic agent.

MATERIALS AND METHODS

Colony-Forming Assays

Mononuclear cells from peripheral blood and marrow samples anticoagulated with preservative-free heparin were separated by sedimentation on Ficoll-Hypaque. All assays were performed in triplicate in 0.8% methyl cellulose incubated at 37{\degree}C in 7.5% CO_2; colonies were cultured for 7-10 days. Normal CFU-GM were assayed in modified McCoy's 5A containing 15% calf serum using media conditioned by pokeweed-mitogen-stimulated lymphocytes at a final concentration of 10% as a source of colony-stimulating factor. When nondialyzed fetal calf serum (FCS) was used, aggregates of >40 cells were scored as colonies on day 10; when dialyzed FCS was used, aggregates of greater than 20 cells were scored as colonies on day 10. Normal CFU-TL were assayed using peripheral blood T cells cultured in modified McCoy's 5A medium^{23} containing 10% FCS with phytohemagglutinin (PHA) conditioned medium at a final concentration of 20% and PHA (Burroughs Wellcome, Research Triangle Park, N.C.) at a final concentration of 2 \mu g/ml. Aggregates of >40 cells were counted as colonies on day 7. Leukemic CFU were assayed in MEM alpha medium (GIBCO, Santa Clara, Calif.) using a modification of the technique described by Minden.^{24} Peripheral blood mononuclear cells from 5 patients with circulating blasts (20%-86%) were depleted of sheep red blood cell receptor-bearing cells and then cultured using 10% PHA-conditioned media. Aggregates of >40 cells were scored as colonies on day 7. Colonies were counted using an inverted microscope. Colony composition was assessed by preparing cytocentrifuge slides of colonies picked with capillaries and stained with standard histo-
chemical stains. Colonies were also tested for their ability to form rosettes with sheep red blood cells.

Preparation of Conditioned Media

Pokeweed-conditioned media was prepared by culturing Ficoll-Hypaque-separated peripheral blood mononuclear cells from normal donors in McCoy's 5-A media with 10% FCS and 1% pokeweed mitogen (GIBCO, Santa Clara, Calif.) for 1 wk at 37°C. PHA-conditioned media was prepared in a similar manner using PHA 2 μg/ml. Conditioned media were sterilized by filtration and stored at −20°C.

Cell Lines

Two cell lines were used in these studies. Colony-forming assays were performed as described for CFU-GM above. HL-60 is a cell line derived from a patient with acute promyelocytic leukemia that retains promyelocytic characteristics. K-562 is a cell line derived from a patient with chronic myelogenous leukemia in blast crisis and retains the Ph1 chromosome.

PHA-Stimulated T-Lymphocyte Cultures

Peripheral blood T cells from normal donors were purified and cultured with PHA in a final concentration of 1 μg/ml as described elsewhere. All cultures were set up in triplicate, and cell viability was determined by trypsin blue dye exclusion. Flow cytometry was performed on cells fixed in 70% ethanol in water, stained with ethidium bromide (10 mg/ml) and mithramycin (25 mg/ml) 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 to obtain estimates of the fraction of cells in G0/G1, S, and G2M phase of the cell cycle. Chicken cells were added as an internal standard.32

RESULTS

dThd Sensitivity of Normal CFU-GM and CFU-TL

In vivo dThd is rapidly cleaved by thymidine phosphorylase to thymine, and in patients receiving high-dose infusions of 75 g/sqm/day, the steady-state serum dThd and thymine levels are both in the range of 10−3 M. Figure 1 shows that dThd in excess of 10−3 M was toxic to both normal marrow CFU-GM and normal peripheral blood CFU-TL when these cells were cultured with dThd for 7–10 days. There was a linear relationship between colony survival and the logarithm of the dThd concentration in the range 10−6–10−3 M for both types of cells, and CFU-GM and CFU-TL were equally sensitive to dThd. In contrast, thymine was not at all toxic to GFU-GM even at 10−3 M; thymine was not tested against T colony-forming cells, but was not toxic at 10−3 M to a number of human cell lines in culture (Streifel J, Howell SB, unpublished observations).

dThd-induced inhibition of cell proliferation is proportional to the depletion of intracellular deoxycytidine triphosphate (dCTP) pools that results from blockade of ribonucleotide reductase. The toxicity of dThd is thus related both to the endogenous intracellular dCTP pool and to the availability of deoxycytidine (dCyd) that can be converted to dCTP via the pyrimidine salvage pathway. FCS is known to contain large amounts of some nucleosides. Two techniques were used to exclude the possibility that dCyd in the FCS was modulating the fractional survival of CFU-GM and CFU-TL cultured with dThd. In one set of experiments, the FCS was extensively dialyzed to completely remove dCyd, and in another set, excess dCyd was added to the cultures. Figure 2 shows that the removal of dCyd by dialysis increased the toxicity of dThd to CFU-GM slightly but did not affect toxicity to CFU-TL. This effect of dialysis could not be attributed to removal of dCyd, since the addition of dCyd in initial concentrations even as high as 10−3 M did not decrease dThd toxicity to CFU-GM (data not shown). This was probably related to rapid conversion of dCyd to deoxyuridine and deoxythidylate to deoxyuridylate by deoxycytidine and deoxythidylate deaminase present in the cultures.

dThd Sensitivity as a Function of Proliferative Rate

The proliferative rate of tissue is one of the determinants of its sensitivity to cell-cycle phase-specific chemotherapeutic agents. To examine the effect of dThd on normal human T cells in different proliferative states, peripheral blood T cells were cultured with PHA, and dThd 10−3 M was added either on day 0, before PHA-induced lymphoblast proliferation had started, or on day 3 at a time when proliferation was well underway. The effect of dThd on the distribution
of cells in various phases of the cell cycle was monitored by flow cytometry, and the changes in the rate of cell death by exclusion of vital dye (trypan blue). The results indicate that whether dThd was cytostatic or cytotoxic was a function of the proliferative state of the T cell before dThd exposure. Figure 3 shows that when dThd was added to cultures on day 0, the major effect was cytostatic: the increase in cell number in the dThd-exposed cultures was significantly slower than in control cultures, but there was no difference in cell viability. In contrast, when dThd was added on day 3 of the culture, it produced much more cell death: there was no increase in the absolute number of viable cells, and the proportion of dead cells was twice as great in the dThd-treated cultures. Other experiments demonstrated that these effects on proliferation rate and cell viability were dependent on dThd concentration in the range of 0.3-3.0 mM and were reversible for at least 24 hr after the start of exposure to 1 mM dThd (data not shown).

The variable effect of dThd as a function of the preexposure proliferative state was also evident in the dThd-induced changes in cell-cycle distribution (Fig. 4). In control cultures, the proportion of the cells in S phase rose rapidly during the first 5 days, peaked in the range of 50%, and then declined as the culture aged further. The proportion of cells in G2M inversely reflected the changes in the fraction of cells in S. The number of cells in G1M rose to an early peak of 20% on day 3 and then declined as the culture matured. When dThd was added on day 0, the accumulation of cells in S phase at the expense of G1 was more pronounced (61% at peak) and sustained, and the rise in the fraction in G2M was blunted and delayed. These changes are consistent with a marked slowing of the transit of cells through S phase, but indicate that despite the presence of 10^{-3}M dThd, some cells were continuing to successfully pass through S and enter G2M. The addition of dThd on day 3 resulted in even more pronounced effects on cell-cycle phase distribution: there was a striking and rapid accumulation of cells in S phase, which peaked at 73% within 24 hr of the time dThd was added, accompanied by a lower nadir of the proportion of cells in G2M; dThd abruptly decreased the fraction of cells in G1M. This pattern of changes suggests that when the T-cell population was already proliferating rapidly at the time the dThd exposure started, there was a virtually complete blockade of cell transit through S. The excess cell death rate in cultures treated with dThd on day 3, when compared to those receiving dThd on day 0, was probably due to death of cells blocked in S or at the G1/S interface. The cell-cycle phase distribution changes could not be attributed to changes in the dThd concentration in the culture media with time. When the initial dThd concentration was 10^{-3}M, it declined only 0.07 log units in 4 days and 0.2 log units in 7 days of culture.
DISCUSSION

*dThd Sensitivity of Leukemic CFU*

Six samples of leukemic blasts were obtained from the peripheral blood of 5 patients with various subtypes of ANLL and tested for their sensitivity to *dThd* in the colony formation assay. In addition, *dThd* was tested against cell lines HL-60 and K-562, derived from patients with ANLL and chronic myelogeneous leukemia in blast crisis, respectively. Figure 5 shows the fractional survival of leukemia colonies as a function of *dThd* concentration compared with the mean survival of normal peripheral blood CFU-GM from three subjects. In contrast to the highly reproducible pattern of sensitivity of normal CFU-GM, there was great variability in the sensitivity of leukemic CFU. Three of the leukemic patient samples, and both leukemic cell lines, were significantly more sensitive than normal CFU-GM over at least part of the *dThd* concentration range, whereas three other leukemic samples were significantly less sensitive to *dThd*. All patients had been extensively treated prior to study, except the patient with monocytic leukemia. Leukemic blasts obtained from this patient before treatment were very sensitive to *dThd*. In contrast, after two courses of chemotherapy with ara-C and 6-thioquanine, this patient's leukemic blasts had become highly resistant to *dThd*, demonstrating greater than 60% survival in the presence of $10^{-3}M$ *dThd*. These results indicate that there is a broad spectrum of *dThd* sensitivity among different patients with ANLL and that the selective pressure of chemotherapy may modify *dThd* sensitivity enormously.

*dThd* was initially reported to cause regression of human tumor xenografts growing in nude mice with minimal toxicity to the host. Serum *dThd* concentrations in the range of $10^{-3}M$ were required for antitumor activity, and subsequent studies from this laboratory demonstrated that at this concentration, *dThd* was toxic to mouse marrow. The studies reported here demonstrated that *dThd* is also toxic to progenitor cells of two normal tissues of the human
body: the bone marrow and the T-lymphocyte population. The magnitude of the reduction in CFU-GM caused by sustained exposure to $10^{-3}M$ dThd is comparable to that produced by clinically effective concentrations of cytosine arabinoside, which routinely cause myelosuppression in patients (Streifel J, Howell SJ, unpublished observations). In corroboration of these findings, myelosuppression has been observed in preliminary trials of high-dose dThd in man. Although not yet reported in patients, the nearly identical dThd sensitivity of CFU-GM and CFU-TL make it likely that prolonged exposure to $10^{-3}M$ dThd will be immuno suppressive to some degree. The mechanism of dThd-induced cytotoxicity involves inhibition of ribonucleotide reductase by high levels of intracellular dTTP. The observations that dThd was toxic to normal human tissues, whereas thymine was not, is consistent with the fact that dThd is readily incorporated into the dTTP pool, whereas thymine is predominantly catabolized to b-aminoisobutyric acid and is a poor precursor of DNA thymine.

Our results strongly suggest that the rate of proliferation of T cells prior to their exposure to dThd influences the ability of these cells to replicate and survive in the presence of dThd. Where exposure was initiated prior to the start of PHA-induced T-cell proliferation, the major effect of dThd was cytostatic. The rate of population doubling was slowed, but production of viable cells continued, and the death rate was not increased even when the proliferative response was well developed. On the other hand, when T cells that were already responding to PHA were exposed to the same concentration of dThd, the major effect was cell killing, and there was no net production of viable cells. This difference in the effect of dThd as a function of preexposure proliferative rate was demonstrable across the dThd concentration range of 0.3–3.0 mM. Cells exposed prior to the start of the PHA-induced proliferative response were apparently able to make protective adjustments to the high dThd environment so that they were subsequently able to survive progression through the cell cycle. A biochemical basis for this adjustment is suggested by studies of temporal changes in the dTTP pool size in leukemic T cells exposed to high concentrations of dThd. Although there was an initial marked accumulation of dTTP, the expansion of the pool size was not maintained and had returned much of the way toward normal by 24 hr. dTTP modulates the activity of several of the enzymes involved in its own synthesis. If the cell is in a resting state when exposed to dThd, it may have time to reduce dTTP to tolerable levels or increase dCTP levels before initiation of DNA synthesis; whereas a cell already committed to DNA synthesis may not.

Although PHA-induced proliferation of T cells represents a type of clonal expansion, the sensitivity of the whole population of responding T cells may not be an accurate reflection of the sensitivity of T colony-forming cells. The concept that preexposure proliferative rate affects the outcome of dThd treatment will require confirmation, and further studies are needed to evaluate the importance of this parameter for other types of cells. A number of investigators recently reported that leukemic T cells are significantly more sensitive to dThd than human B-cell lines. The basis of this sensitivity has been ascribed to deficiency of enzymes in the dTTP catabolic pathway in T cells. In contrast, our results indicate that normal human T cells display dThd sensitivity quite similar to that of marrow colony-forming cells, suggesting that there are not major differences in the patterns of dThd metabolism between CFU-GM and CFU-TL.

Our studies of leukemic CFU demonstrate a broad spectrum of dThd sensitivity among blasts freshly harvested from patients with various forms of ANLL and two leukemic cell lines. Although half of the samples from this small group of patients were less sensitive to dThd than normal CFU-GM, for the other samples the concentration of dThd required for 50% inhibition of colony formation was a minimum of two orders of magnitude less than that required to inhibit normal CFU-GM, suggesting that in this latter group of patients, dThd might prove highly selective in vivo. These studies also provide some evidence that the proliferative state of the progenitor cells prior to dThd exposure, and prior exposure of the cells to chemotherapeutic agents, may contribute to this rather wide variation in dThd sensitivity. In this regard, the patient with acute monocytic leukemia was of particular interest: leukemic cells from this patient were very sensitive to dThd when first tested at the time of diagnosis ($ID_{50} < 10^{-7}M$), but after a single course of chemotherapy containing ara-C the blasts had become highly resistant ($ID_{50} > 10^{-3}$). In vitro studies have shown that resistance to both ara-C and dThd can develop concurrently as the result of a single one-step biochemical alteration in the endogenous dCTP pool size.

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