Pharmacologic Marrow Purging in Murine T Cell Leukemia

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Deoxycoformycin in combination with deoxyadenosine was used to purge 6C3HED malignant T cells from murine marrow in vitro. Adenosine deaminase activity of 6C3HED cells was ablated by incubation with \(10^{-8}\) mol/L deoxycoformycin (dCF). During a 12-hour incubation with \(10^{-6}\) mol/L dCF and \(10^{-4}\) mol/L deoxyadenosine, tumor cells sequentially accumulated dATP, became depleted of NAD followed by ATP, then died. More than 5 logs of 6C3HED marrow in vitro. Adenosine deaminase activity of 6C3HED was used to purge 6C3HED malignant T cells from murine marrow. In a rodent model, the limited availability of deoxyadenosine (dAdo), which is essential to the effectiveness of dCF in vitro is enhanced by the provision of additional dAdo. Malignant T cells that were not susceptible to dCF alone and did not accumulate dATP in vitro or in vivo in the absence of additional dAdo, did accumulate dATP when exogenous dAdo was added in vitro. Thus, the limited availability of dAdo in vivo may significantly limit the effectiveness of systemic treatment with dCF. We report the development of a murine model for purging malignant T cells from marrow in vitro using dCF in combination with dAdo.

MATERIALS AND METHODS

**Animals**

Female C3H/HeN mice 6 to 8 weeks old were obtained from Harlan-Sprague Dawley, Indianapolis. They were housed four to six per cage in an enclosed laminar-flow, filtered-air system (BioClean Hazelton Systems, Aberdeen, MD) and provided with food and water ad libitum.

**Tumor Cells**

The Gardner 6C3HED, estrogen-induced, thymus-derived malignant murine T-cell line \(^{(1)}\) (NSC402421) was obtained from Arthur D. Little (Cambridge, MA). Cells were passaged in ascites and maintained in vitro in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mmol/L glutamine.

**In Vitro Treatment**

Tumor cells, bone marrow, or a mixture of the two were incubated with \(10^{-6}\) mol/L dCF and \(10^{-8}\) mol/L dAdo for 12 hours (unless otherwise indicated) at 37°C in a humidified atmosphere containing 5% CO\(_2\). Media pH was not altered following addition of dCF and dAdo. Cells were washed three times with Seligman's balanced salt solution (SBSS) containing 1% EDTA and either resuspended in Hank's balanced salt solution (HBSS) for injection; pelleted for the dATP, ATP, or NAD assay; or resuspended in 5 mmol/L tris, pH 7.4, 0.25 mol/L sucrose for the enzyme assays.

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**SELECTIVE IN VITRO eradication of malignant cells from marrow is essential to the application of autologous marrow transplantation to leukemia and other malignancies that involve the marrow.** Monoclonal antibodies directed against antigens on leukemia cells have been used to purge malignant cells from the marrow of patients with acute lymphocytic leukemia (ALL) of B-cell lineage. An alternative approach for marrow purging uses chemotherapeutic agents that possess great activity against specific malignant cell populations than against hematopoietic precursor cells. The drug 4-hydroperoxycyclophosphamide has been used to eliminate myelogenous leukemia cells from marrow in a rodent model. A preliminary study in humans is encouraging.

Unique purine metabolism in T cells makes them especially susceptible to inhibitors of purine catabolism. Inherited deficiency of the purine degradation enzyme adenosine deaminase (ADA) causes T cell depletion and severe combined immunodeficiency (SCID). ADA catalyzes the deamination of adenosine and 2' deoxyadenosine to inosine and 2' deoxynosine, respectively. Deamination is the chief route of deoxyadenosine metabolism in murine and human tissues. The specific toxicity of this enzyme deficiency for T lymphocytes results from their high levels of activity of deoxyadenosine phosphorylating enzymes relative to deoxyadenosine dephosphorylating enzymes. High intracellular concentrations of dATP are toxic to T cells, initially interfering with T-cell proliferation and eventually causing cell death.

The potent ADA inhibitor deoxycoformycin (dCF) produces biochemical effects in T cells identical to those of inherited ADA deficiency. Although systemically administered dCF is effective treatment for patients with a variety of lymphoid malignancies, its use in ALL has been limited by dose-dependent, extramedullary toxicity. Marrow toxicity has been mild, indicating limited hematopoietic stem cell injury. Malignant cells can be exposed in vitro to concentrations of dCF sufficient to ablate ADA activity. The effectiveness of dCF in vitro is enhanced by the provision of exogenous deoxyadenosine (dAdo), which is essential to the accumulation of dATP and the antileukemic effect of dCF. Malignant T cells that were not susceptible to dCF alone and did not accumulate dATP in vitro or in vivo in the absence of additional dAdo, did accumulate dATP when exogenous dAdo was added in vitro. Thus, the limited availability of dAdo in vivo may significantly limit the effectiveness of systemic treatment with dCF. We report the development of a murine model for purging malignant T cells from marrow in vitro using dCF in combination with dAdo.

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Transplantation of Marrow

Donor bone marrow was flushed from femurs of female mice, filtered through sterile gauze, centrifuged, and resuspended in either HBSS for injection or supplemented RPMI for treatment. Recipient male mice were treated 18 to 24 hours prior to transplantation with 180 through sterile gauze, centrifuged, and resuspended in either filtered HBSS, or a combination was injected into the tail vein.

Spleen Cell Colony Determination

Irradiated recipient mice were killed 10 days after transplantation. The spleens were removed and fixed in Bouin's solution. The number of discrete colonies was counted. The number of mice surviving at day 10 for determination of CFU-S compared to the number transplanted were as follows: untreated—1 x 10⁶ (9 of 10), 5 x 10⁵ (8 of 10), 1 x 10⁵ (9 of 12); treated—1 x 10⁶ (10 of 10), 5 x 10⁵ (9 of 15), 1 x 10⁵ (11 of 25).

Enzyme Extraction

Ten to 15 x 10⁶ cells in 0.5-mL vol were sonicated in 15-second bursts for 2 minutes at 4°C. Cellular debris was removed by centrifugation at 50,000 g at 4°C for 30 minutes. Samples were stored at —20°C for 1 to 5 days.

ADA

The reaction was initiated with the addition of 20 µL sample to 50 µL reaction mixture containing 8-¹⁴C-adenosine (60 mCi/mmol, Amersham, Arlington Heights, IL), 1.99 mmol/L adenosine, and 0.23 mol/L 0.4 mol/L Tris-HCl buffer in a shaking 37°C waterbath. The assay was terminated after 10 minutes by the addition of 20 µL 4 mol/L formic acid at 4°C. A 5-µL sample was placed on cellulose acetate thin-layer chromatography plates to which unlabeled markers had been previously applied. After developing in water for 1 hour, the bands were cut out and the amount of radioactivity was determined by thin-layer chromatography.

5'-Nucleotidase Assay

The reaction was initiated with the addition of 30 µL enzyme extract to 30 µL reaction mixture containing ¹⁴C-monomophosphate (56 mCi/mmol, Amersham) 200 µmol/L inosine monophosphate, 50 mmol/L MgCl₂, 100 mmol/L Tris-acetate, and 2.5 mmol/L Tris-HCl in a 37°C shaking waterbath. The reaction was terminated after 15 minutes by placing the tube in a 95 to 100°C waterbath for 2 minutes. The amount of radiolabeled product was determined by thin-layer chromatography as in the ADA assay. Activity is expressed as 10⁻⁷ mol inosine formed/h/10⁶ cells.

Deoxyadenosine Kinase Assay

Deoxyadenosine kinase assay was performed similarly to the 5'-NT assay except that 20 µL sample was added to 40 µL reaction mixture containing ¹⁴C-dAdo (55 mCi/mmol, Dupont/New England Nuclear, North Billerica, MA), 100 µmol/L dAdo, 5 mmol/L ATP, 2 mmol/L dithiothreitol, 20 mmol/L MgCl₂, and 100 mmol/L Tris-HCl. The reaction was terminated after 30 minutes. Activity is expressed as 10⁻⁷ mol adenosine monophosphate formed/h/10⁶ cells.

Extraction of NAD

Pellets containing 7 to 12 x 10⁶ cells were incubated in 0.5 mL 0.5 mol/L perchloric acid for 15 minutes at 4°C. After the samples were adjusted to pH 7.5 using 0.5 mL 1 mol/L KOH/0.33M KH₂PO₄, insoluble KClO₄ was removed by centrifugation at 2,200 g for 5 minutes at 4°C. Supernatants were stored at —20°C for 1 to 5 days.

NAD Cycling Assay

NAD was measured by placing 0.2 mL cell extract, 0.1 mL alcohol dehydrogenase (160 U/mL, Sigma Chemical, St Louis), and 1.0 mL reaction mixture containing 600 mmol/L 100% ethanol, 0.5 mmol/L methyl thiazolyl tetrazolium (MTT), 20 mmol/L phenazine ethosulfate, 5.0 mmol/L ethylenediamine tetraacetic acid, 1 mg bovine serum albumin (BSA), and 120 mmol/L N-bis (2-hydroxyethyl) glycine (pH 7.8) at 37°C in the dark. After 20 minutes, the reaction was terminated by the addition of 0.5 mL 12 mmol/L iodoacetate. The optical density of reduced MTT was measured using a spectrophotometer at 570 nm. The amount of NAD is expressed as pmol/10⁶ cells.

Measurement of Intracellular Nucleotides

Extraction of dATP and ATP. Fifty microliters sodium triphosphate was added to pellets containing 7 to 12 x 10⁶ cells. Three milliliters of 60% methanol was added dropwise. Samples were frozen overnight at —70°C. Following centrifugation at 10,000 g for 2 minutes, methanol was removed by passing nitrogen over the samples at 30°C. The remaining water was evaporated using a Meyer N-EVAP Analytical Evaporator (Organamation, South Berlin, MA). Two hundred-fifty microliters distilled water was added, and the samples were centrifuged at 10,000 g for 2 minutes. Samples were stored at —70°C for 1 to 5 days.

dATP Assay. The reaction was initiated by the addition of 25 µL sample to 100 µL reaction mixture containing 50 mmol/L Tris-HCl, pH 7.8, 5 mmol/L MgCl₂, 1 mmol/L β-mercaptoethanol, BSA 50 µg/mL, 20 µg/mL primer template (poly (dA-T)), 0.5 mmol/L dATP, 1.5 U/mL DNA polymerase and 10 µmol/L dNTP in a 37°C shaking water bath. The reaction was terminated after 60 minutes by addition of 100 µL assay mixture to fiber filter strips and three washes in cold 5% TCA containing 1% sodium pyrophosphate. The strips were then washed three times in 95% ethanol. The amount of radiolabeled product was determined using a Packard liquid scintillation counter. The amount of dATP present is expressed as pmol/10⁶ cells.

ATP. ATP was determined by high-performance liquid chromatography (HPLC) using ion-exchange chromatography with 0.45 mmol/L NH₄H₂PO₄ buffer, an Ultex Ultrasil-Ax column, flow rate 1 mL/Min, and ultraviolet detector at 254 nm. ATP values are expressed as pmol/10⁶ cells.

Reagents
dCF was obtained from the Investigational Drug Branch of the National Cancer Institute, Bethesda, MD. dAdo was purchased from Sigma. Other chemicals were of the highest quality commercially available.

Statistical Analysis

The differences between survival patterns were evaluated by Yates corrected chi-square test.

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RESULTS

Enzyme Levels in 6C3HED Cells

The ADA level of 6C3HED cells was $1.14 \pm 0.06 \times 10^{-4}$ mol inosine formed/h/10^6 cells. The deoxyadenosine kinase (dAdoK) level was $1.15 \pm 0.09 \times 10^{-7}$ mol adenosine monophosphate formed/h/10^6 cells, and the 5'NT level was $1.86 \pm 0.55 \times 10^{-7}$ mol inosine formed/h/10^6 cells. These values represent the mean and SD of three separate determinations. During incubation of 6C3HED cells with $10^{-4}$ mol/L dCF, the ADA level was zero as measured at 15 minutes, 6 hours, and 12 hours in three separate experiments.

Metabolic Studies During Incubation of 6C3HED Cells With dCF/dAdo In Vitro

Figure 1 shows sequential metabolic studies during incubation of 6C3HED cells in $10^{-4}$ mol/L dCF and $10^{-4}$ mol/L dAdo. Intracellular accumulation of dATP occurred within 30 minutes (Fig 1A). The accumulation of large amounts of dATP was followed by progressive depletion of NAD and ATP (Fig 1B). After 12-hour incubation, no viable cells (by trypan blue dye exclusion) remained. To exclude the possibility that decreased cell viability was largely responsible for NAD and ATP depletion, Ficoll-Hypaque gradient centrifugation was used to enrich for viable cells. Following 8-hour exposure to dCF and dAdo and then Ficoll-Hypaque centrifugation, viability of collected cells was 78%. The NAD level in these cells was 22.8% control (untreated cells), and the ATP level was 27.2% control levels, demonstrating that NAD and ATP depletion precede loss of cell viability.

Effectiveness of 6C3HED Cell Kill by dCF/dAdo

The dose of 6C3HED cells required to kill normal C3H/HeN mice was determined. Intravenous (IV) injection of as few as 25 viable cells killed 9 of 14 mice (64%) (Fig 2A). Injection of $\geq$250 cells killed 100% of mice. Next, the cytotoxic effectiveness of incubation in dCF and dAdo was tested. Various doses of 6C3HED cells were incubated in $10^{-4}$ mol/L dCF and $10^{-4}$ mol/L dAdo for 12 hours and then injected IV into C3H/HeN mice. Sixteen of 18 mice from three separate experiments, injected with $2.5 \times 10^6$ treated tumor cells, were survivors at 3 months (Fig 2B). All 18 mice injected with $2.5 \times 10^6$ tumor cells were survivors. Seventeen of 18 mice injected with $2.5 \times 10^6$ tumor cells survived. All mice injected with $2.5 \times 10^5$, $2.5 \times 10^4$, and $2.5 \times 10^3$ treated tumor cells were survivors at 3 months. In contrast, the survival of mice injected with tumor cells incubated for 12 hours without dCF/dAdo was virtually identical to that of mice injected with unincubated tumor cells. The survival curves of mice injected with $2.5 \times 10^5$ or $2.5 \times 10^6$ tumor cells are displayed in Figure 2B. All 18 mice injected with $2.5 \times 10^5$ 6C3HED cells incubated without dCF/dAdo died within 16 days of injection. Survival curves similar to those shown in Fig 1A were generated by inoculation of smaller numbers of tumor cells that had been incubated in the absence of dCF and dAdo.

Toxicity of Incubation With dCF/dAdo on Hematopoietic Precursors

Table 1 shows the number of CFU-S observed 10 days following injection of various doses of treated or untreated marrow cells into irradiated C3H/HeN mice. The 2-month survival of mice injected with marrow cells in two separate experiments is also shown. An approximately tenfold reduction in hematopoietic progenitors is indicated by both CFU-S and 2-month survival data. Transplantation of $5 \times 10^5$ treated marrow cells rescued 11 of 12 lethally irradiated mice.

Transplantation of Treated Leukemic Bone Marrow

Figure 3 shows the survival of irradiated mice rescued with leukemia-contaminated marrow that had been incubated with dCF and dAdo. Twelve of 14 mice were long-term survivors. Six animals were randomly killed at 6 months and the remainder were killed at 9 months. All demonstrated
Histologically normal marrows with no evidence of leukemia. Two mice died within 2 weeks of transplantation and demonstrated early evidence of engraftment with no histologic evidence of leukemia. All 14 mice injected with untreated marrow contaminated with 5% leukemic cells died within 2 weeks of transplantation, as did all irradiated controls (animals not rescued with marrow). There was a significant difference in survival between the group of mice receiving untreated leukemic marrow and the group receiving treated leukemic marrow ($P < .01$). Autopsies of animals injected with untreated leukemia cells demonstrated widespread leukemia. Infiltration of the bone marrow and spleen were most prominent.

**DISCUSSION**

In the murine model we developed, >5 logs of malignant T cells were killed by concentrations of dCF and dAdo that had relatively modest effects on hematopoietic progenitor cells. Preliminary studies designed to examine the effectiveness of tumor cell kill revealed that a 12-hour incubation with dCF and dAdo killed ten times the number of tumor cells that were later used to contaminate normal marrow in transplantation experiments. Investigation of the effect of incubation in dCF and dAdo on normal hematopoietic progenitor cells demonstrated engraftment of treated marrow cells with one tenth the marrow inoculum used in transplantation experiments. This differential activity of dCF in combination with dAdo permitted the selective eradication of malignant cells from marrow ex vivo. The use of marrow contaminated with 5% leukemia cells in transplantation experiments was designed to imitate the marrow aspect of patients with acute lymphocytic leukemia in complete remission.

To define the mechanism of cell death, the activities of relevant enzymes of purine metabolism were measured and the sequential metabolic alterations in leukemic cells incubated with dCF and dAdo were determined. The 6C3HED malignant T-cell line by virtue of a relatively low (for T cells)
ADA level and high ratio of dAdoK as compared with 5’NT activity (characteristic of T cells) represented an ideal tumor for study. 6C3HED cells are susceptible to ablation of ADA activity with a low concentration of dCF because of their relatively low ADA activity.13 When ADA activity is ablated, the relative activity of dAdoK as compared with 5’NT determines the rapidity and degree of dATP accumulation. Evidence that a phosphorylated form of dAdo is important in toxicity is provided by cell mutants that are unable to phosphorylate ATP, leading to depletion of cellular NAD pools.24 The metabolic studies suggest that following incubation of malignant murine cells in dCF and dAdo, dATP accumulation is followed by NAD and ATP depletion. This sequence of events has been previously identified in normal human lymphocytes.25

These observations suggest a mechanism of cell death similar to that proposed for ADA-deficient T cells and for normal human T cells exposed to dCF.25 Poly(ADP-ribose) polymerase, activated by dCF-induced DNA strand breaks, cleaves NAD, leading to depletion of cellular NAD pools. NAD depletion interferes with ATP synthesis. Depletion of ATP results in loss of all energy-dependent functions, including glucose utilization, DNA, RNA, and protein synthesis and maintenance of membrane integrity.26 Cell death follows. The rapidity and completeness of cell kill reported in this study exceed that previously reported and are likely related in part to the drug concentrations used and in part to the enzyme profiles and relative homogeneity of the 6C3HED cells. The ability to measure the levels of enzymes responsible for dATP accumulation and to monitor the relevant metabolic events may permit prediction of the susceptibility of specific malignancies to this particular treatment strategy. It also provides the potential for enhanced chemotherapeutic efficiency, eg, by agents that inhibit synthesis of NAD.

Effective killing of malignant T cells in vitro by dAdo and dCF has generally been measured by 3H-thymidine incorporation and clonogenic assays.27,28 These studies have suggested a potential role for dCF and dAdo in purging marrow of malignant T cells; however, these in vitro studies should be interpreted cautiously. Elevated dATP levels inhibit ribonucleotide reductase, which catalyzes the biosynthesis of deoxyribonucleotides from ribonucleotides.29 Inhibition of this critical step in DNA synthesis may result in markedly diminished 3H thymidine uptake and limit the clonogenicity of cells, but does not cause rapid cell kill. The limitation of in vitro culture systems in this context has been previously documented. Incubation of rodent and human marrow with a drug such as 4-hydroperoxycyclophosphamide eliminated granulocyte-macrophage colony forming cells without preventing hematopoietic reconstitution.24 In vivo animal models are therefore particularly important for study of pharmacologic marrow purging, especially with dCF and dAdo.

In summary, we developed a murine model for purging malignant T cells from marrow and accumulated preliminary information on the biochemistry of cell death. This animal model provides information that cannot be obtained by previous in vitro systems. The application of this model to other murine T-cell malignancies and to human T-cell neoplasms is currently being investigated.

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PHARMACOLOGIC MARROW PURGING

1661


Pharmacologic marrow purging in murine T cell leukemia

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