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THE FRIEND MURINE ERYTHROLEUKEMIA CELL, A MODEL SYSTEM FOR STUDYING THE ASSOCIATION BETWEEN BONE MARROW TOXICITY INDUCED BY 3'-AZIDO-3'-DIDEOXYTHYMIDINE AND DIDEOXYNUCLEOSIDE INHIBITION OF mtDNA REPLICATION

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Major toxicities of ddNs[+] in AIDS therapy (see Ref. 1 for citations) include AZT-induced macrocytic anemia, myopathy, and cardiomyopathy, ddC- and ddI-induced peripheral neuropathy, and ddI-induced pancreatitis. The dd-derivatives of the naturally occurring precursors of DNA are excellent substrates for DNA polymerase gamma, becoming incorporated into mtDNA and inhibiting DNA replication by chain termination (see Ref. 1 for citations). It thus seemed reasonable to propose that unnatural dd-analogs such as AZT might act similarly (1). The resultant inhibition of mtDNA replication, either by chain termination or possibly in some cases by competitive inhibition, should have serious consequences for mt biogenesis, mt function and in turn for cell function and viability. This view was supported by studies on isolated rat mitochondria showing that anti-HIV-1 ddNs, in particular AZT, inhibit mtDNA replication severely (1). Subsequent studies (2) demonstrated that anti-HIV-1 ddNTPs including AZTTP, inhibit DNA replication by polymerase gamma. In addition, a preliminary investigation using the Friend murine erythroleukemia cell, selected as a model for studying AZT-induced anemia, showed that twelve anti-HIV-1 ddN analogs exerted a range of inhibitory effects on cell growth, correlating well with the results on isolated mitochondria. Moreover, mitochondria isolated from AZT-grown Friend cells were impaired in DNA replication.

The incubation conditions in these early experiments (2% DMSO, moderate aeration), although commonly employed for these cells, are suboptimal for maximum cell growth, possibly resulting in drug hypersensitivity and inaccurately reflecting in vivo toxicity. Under our new and improved conditions, of the twelve analogs (+ddC), AZT was the only one which inhibited Friend cell proliferation; it is also the only one of these known to strongly suppress bone marrow. This selectivity is highlighted by our recent finding that, while ddC, d4T and ddI exert deleterious effects on the PC12 cell (a model for a peripheral neuron), reflecting their relative propensity to induce peripheral neuropathy, AZT is without effect (3). We describe here these and the following new results. We have asked whether AZT-induced anemia might result from specific inhibition of hemoglobin synthesis, but our results do not support this view. Finally, although our detailed studies on the metabolic consequences of the AZT inhibition of mtDNA replication will be described elsewhere, one unexpected finding, the triggering by AZT of a substantial increase in the mt content of the Friend cell, is reported here.

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[+]Abbreviations: dd, dideoxy; ddN, dideoxynucleoside; ddNTP, dideoxynucleoside triphosphate; AZT, 3'-azido-3'-deoxythymidine; AZTTP, 3'-azido-3'-deoxythymidine triphosphate; ddC, dideoxycytidine; N_3-IUdR, 3'-azido-2', 3'-dideoxy-5-iodouridine; N_3-BUdR, 3'-azido-2', 3'-dideoxy-5-bromouridine; An-N_3-IUdR, 2,5'-anhydro-3'-azido-2', 3'-dideoxy-5-iodouridine; An-N_3-BUdR, 2,5'-anhydro-3'-azido-2', 3'-dideoxy-5-bromouridine; N_3-UdR, 3'-azido-2', 3'-dideoxyuridine; d4T, 3'-deoxythymidin-2'-ene; An-N_3-TdR, 2,5'-anhydro-3'-azido-3'-deoxythymidine; 5-Me-d4C, 2', 3'-dideoxy-5-methylcytidin-2'-ene; An-N_3-UdR, 2,5'-anhydro-3'-azido-2', 3'-dideoxyuridine; ddI, dideoxyinosine; d4C, 2', 3'-dideoxycytidin-2'-ene; AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; mt, mitochondrial; DMSO, dimethyl sulfoxide; NAO, N-nonyl acridine orange; and PBS, phosphate buffered saline.

MATERIALS AND METHODS

Uninduced Friend cell stocks were grown in 25 mL canted neck flasks in medium A (RPMI 1640, penicillin, streptomycin, and 10% bovine/5% fetal bovine serum). For growth experiments, seeding was at 1.8-2.0 x 10^5 cells/mL in Falcon polypropylene round bottom tubes (17 x 100 mm) containing medium B (5 mL medium A, 1.8% in DMSO, plus the ddN). The loosely capped tubes were drum rotated (6 rpm, 80° angle) at 37° in humidified air/5% CO_2. Passage was at 1-2 x 10^6 cells/mL in medium B. Hemoglobin was determined spectrophotometrically (4). For mt isolation, cells were grown in 100 mL spinner flasks, the PBS-washed pellet was resuspended in hypotonic solution [10 mM Tris (pH 7.1), 10 mM KCl, 150 μM $MgCl_2$] for 15 min, the suspension was homogenized and mitochondria were isolated, incubated with [^{3}H]dATP, precipitated and counted (1). Mitochondria/cell was determined from the protein content of mt pellets, from the extent of binding (5) of the mt-specific dye, NAO (measured spectrofluorimetrically on cells washed three times with PBS), and from electron microscopy of 31 randomly selected cell sections in two independent experiments, each done in triplicate.

RESULTS AND DISCUSSION

Our preliminary studies (2), done under conditions found later to be suboptimal for Friend cell replication, showed that of twelve ddNs studied, six, led by AZT, were strongly inhibitory. Under current conditions (1.8% DMSO, improved aeration) doubling time decreased more than 30%, and DMSO no longer inhibited cell growth immediately upon addition. Under these conditions, of thirteen anti-HIV-1 analogs tested at 25 μM, only AZT and ddC inhibited, strongly and weakly, respectively (Fig. 1). At 5 μM, even the ddC inhibition disappeared (Fig. 2). Notably, of the analogs among these for which clinical information is available (ddC, ddI, d4T, N_3-UdR, AZT), only AZT and N_3-UdR are known to suppress bone marrow, the latter only weakly. These results are remarkably complementary to those on the PC12 cell which show that cell growth, neurite development and neurite survival are adversely affected only by analogs that induce peripheral neuropathy (ddC, ddI, d4T) but not by AZT (3). The results point to the potential of the paired systems for toxicity, tissue selectivity, and drug screening studies.

Might anemia result from a direct effect of AZT on hemoglobin synthesis? In one experiment (Fig. 3A), DMSO was added at the beginning of the incubation while AZT was added later but prior to cessation of cell growth, so that there would be a minimal effect on differentiation but cell growth could still be measured. In the second experiment, AZT was added almost at the cessation of cell growth and about the point at which hemoglobin synthesis begins (Fig. 3B). The results in Fig. 3A show that at the 9-day point, 5 μM AZT decreased cell growth and hemoglobin/cell 7.8 and 3.0% respectively; at 25 μM, the values were 18.1 and 3.8%. The 10-day point (at which time some cell death had occurred) yielded values of 8.8 and 5.6%, respectively, at 5 μM AZT, and 14.7 and 14.6%, respectively, at 25 μM AZT. Under conditions of cessation of cell growth, with therefore little possible effect of AZT on this parameter, AZT had no effect on hemoglobin synthesis (Fig. 3B). These results offer little support for a direct effect of AZT on hemoglobin synthesis. More likely, AZT acts on the hemopoietic progenitor cell as it does on the Friend cell, by inhibition of mtDNA replication and mt function.

Preliminary results showed depressed mtDNA replication in mitochondria isolated from AZT-grown cells (2). Under our new conditions and at a lower (1 μM) AZT level, after adjustments for both fewer cells and increased mitochondria/cell in the AZT samples, the extent of mtDNA replication per mitochondrion showed a decrease of 48.3% (Table 1). This reduction may have two components: First, the amount of mtDNA (i.e. the number of DNA templates) per mitochondrion is 30% less in the AZT cells (manuscript in preparation). Second, this value means that the specific activity of the mtDNA is 26% less in the AZT cells, suggesting impaired replication. The AZT-induced increase in mitochondria/cell shown by three independent methods (Table 2) was about 1.75-fold (based on the EM method, probably the most accurate). Inasmuch as this occurs in the face of inhibition of mtDNA replication, the new mitochondria must have fewer mtDNA molecules, which is in fact the case. These phenomena, including the regulatory mechanism of the mt increase, are currently under investigation.

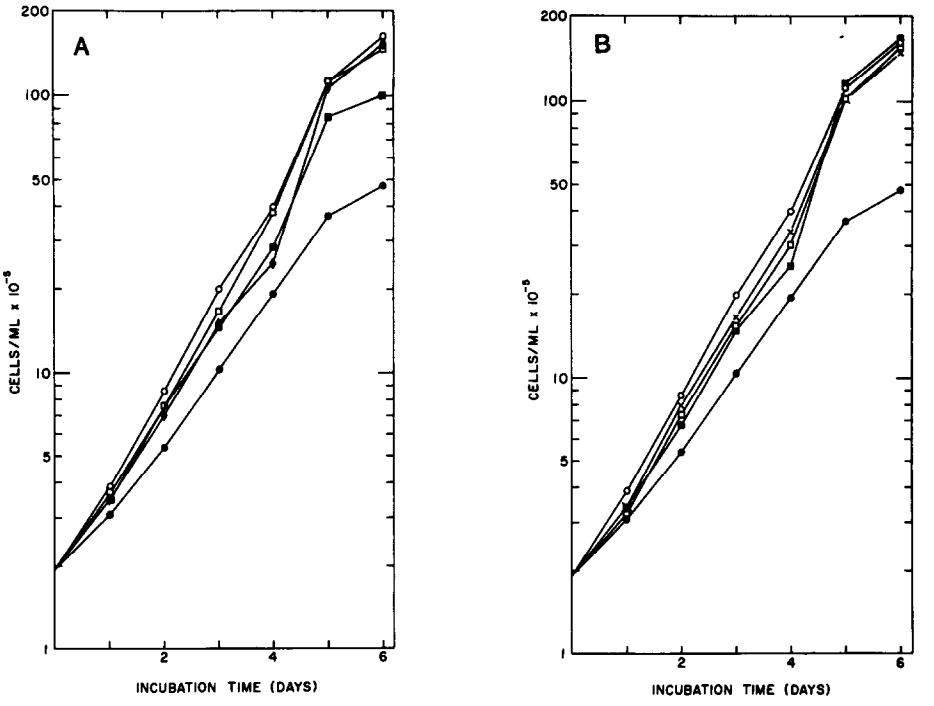

Fig.1. Effect of 25 μM ddN analogs on growth of 1.8% DMSO-induced Friend cells. (A) Control (○), d4T, d4C, 5-Me-d4C (□), ddI (◆), ddC (■) and AZT (●). (B) same as A except N_3-BUdR (□), N_3-UdR, An-N_3-BUdR, An-N_3-IUdR, An-N_3-UdR, N_3-IUdR (X), An-N_3-TdR (■). Experiment is split between two graphs for clarity.

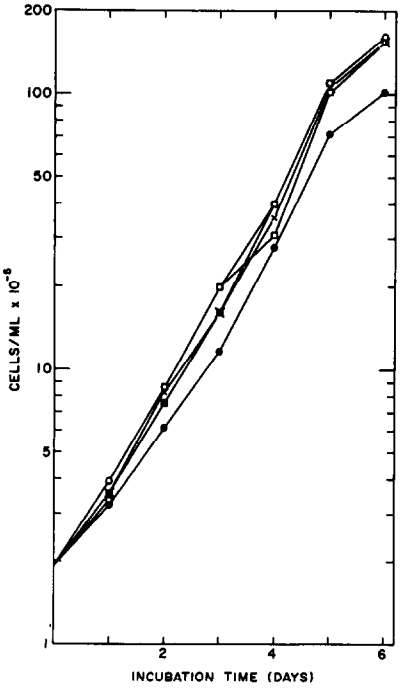

Fig. 2. Effect of 5 μM ddN analogs on the growth of 1.8% DMSO-induced Friend cells. Control, An-N_3-BUdR (○), N_3-BUdR, N_3-UdR, An-N_3-IUdR (X), An-N_3-UdR, N_3-IUdR (□), d4C, ddC, (■), and AZT (●).

Table 1. mtDNA replication in AZT-grown Friend cells

	cpm in Mito.	cpm, Normalized for Cell #[1]	for Mito/Cell[2]	% Inhibition
Control	12600	4040	4040	
1 μM AZT	8620	3650	2090	48.3

[1]Cells harvested x10^6: Control, 3.11, AZT, 2.36.
[2]See text.

Table 2. Effect of AZT on mitochondrial proliferation

Conditions			Fold-stimulation/Cell over no AZT control		
Inducer[1]	AZT μM	Incubation time (hr)	mt Protein[2]	NAO binding[3]	EM[4]
DMSO	1	60-140	1.43(5)		
DMSO	5	48	1.46(5)	1.58	1.75
DMSO	10	43-168	1.66(6)		
Butyrate	5	99	2.04(1)		

[1]DMSO was at either 1.5% or 1.8%; mt protein/cell was not affected by this variation. Butyrate, also a good inducer of Friend cell differentiation was at 1 mM.
[2]Mean values derived from the indicated number of experiments.
[3]Relative fluorescence corrected for background.
[4]Electron microscopy.

Important evidence for mtDNA replication being the primary cellular target of AZT also comes from studies on Molt-4F cells (6) and from recent in vivo studies showing that long-term administration of AZT to patients with HIV infection or to laboratory animals may lead to myopathy or cardiomyopathy associated with ragged red fibers (indictive of abnormal mitochondria) and gross defects in mt structure (7-9).

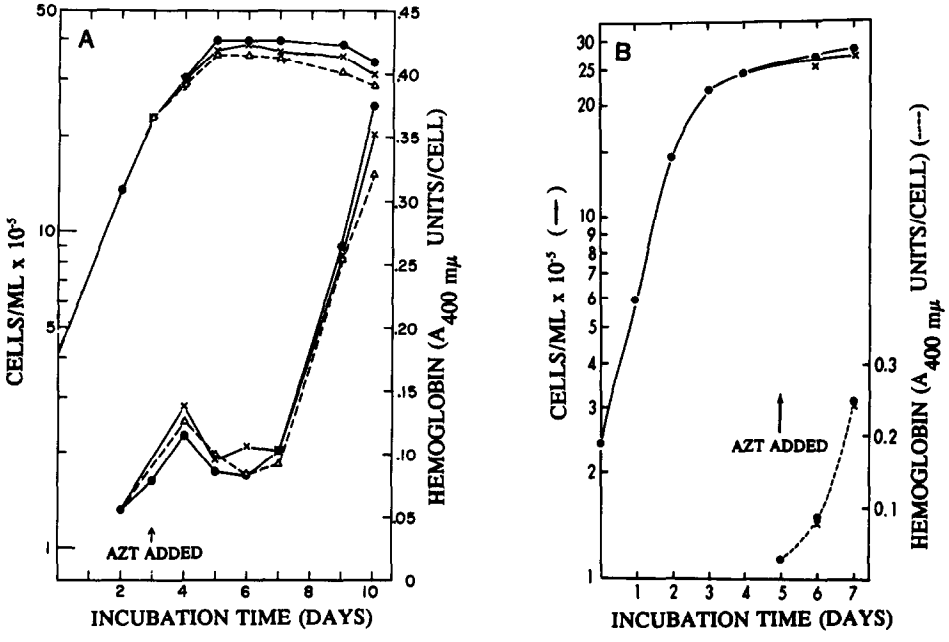

Fig. 3. Effect of AZT on hemoglobin synthesis by the Friend cell. (A) Drug added before growth plateau. Cell growth, upper curves; hemoglobin, lower curves. Peak at 4 days is artifactual. (B) Drug added after growth plateau. Control (●), 5 μM AZT (x), and 25 μM AZT (▲).

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