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CELLULAR TARGETS OF 3′-AZIDO-3′-DEOXYTHYMIDINE: AN EARLY (NON-DELAYED) EFFECT ON OXIDATIVE PHOSPHORYLATION

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Abstract—Previous results demonstrated that incubation of the Friend murine erythroleukemic cell with 5 μM AZT for several days leads to a decrease in the rate of cell growth, inhibition of mtDNA replication, reduction of mtDNA per cell and per mitochondrion, and an increase in mitochondria per cell. As shown here, such treatment also leads to changes in lactate and ATP synthesis and in O_2 uptake, suggesting impairment of oxidative phosphorylation. Direct measurement of ATP synthesis in mitochondria isolated from AZT-treated cells confirmed this view. The most significant new finding in this paper, however, is that in addition to these *delayed* effects of AZT, similar but very rapidly appearing effects on oxidative phosphorylation were noted, with changes observed in the above parameters including mitochondrial proliferation. Some of these occurred as early as 3 hr, only 7% of the doubling time, after exposure of the cells to 5 μM AZT, a period too short for initiation of appreciable mtDNA-mediated effects. Studies on isolated mitochondria provided no evidence of the identity of the immediate target of AZT: AZT does not act as an uncoupler or inhibitor of respiratory control, and previous results failed to implicate adenylate kinase. We have also begun to address the question of the mechanism of AZT-induced mitochondrial proliferation. Initial experiments showed that AZT inhibited synthesis of total cytosolic protein but stimulated synthesis of those proteins imported into mitochondria from the cytoplasm. We also report that aminothymidine, a catabolite of AZT in liver capable of inhibiting cell growth, was not generated by Friend cells.

Key words: AZT toxicity; 3′-azido-3′-deoxythymidine; oxidative phosphorylation; AZT targets; zidovudine toxicity; mitochondria

It is now well established that an important target of anti-HIV-1 ddN§ analogs in the mammalian cell is the mitochondrial enzyme, DNA polymerase γ, and that the consequent inhibition of mtDNA replication can account for at least some of the important toxic side-effects of these analogs. Thus, impairment of mtDNA replication will eventually lead to a depletion of mtDNA. Inasmuch as the mammalian mitochondrial genome encodes 13 proteins, all of which are involved in terminal electron transport/oxidative phosphorylation, long-term exposure of AIDS patients, animals or cells to ddNs would be expected to lead to mitochondrial damage and an inhibition of ATP synthesis with adverse consequences to the cell. Evidence supporting this scenario [1–15] has been summarized recently [16].

AZT toxicity manifests itself primarily in bone marrow suppression and in skeletal and cardiac myopathies. In contrast, the non-azidylated anti-HIV-1 drugs used in AIDS therapy, namely ddC, ddI, and d4T, act primarily on the peripheral nervous system, inducing painful peripheral neuropathy. This differential toxicity is reflected with surprising accuracy in the differential effect of the two drug sets on two cell lines, one, a bone marrow model (the Friend mouse erythroleukemic cell), the other, a model of a peripheral neuron (the PC12 cell) [5].

These selective actions of the two drug sets may be a reflection of their different biochemical properties. Although both drug types inhibit mtDNA replication [3, 4, 6, 10], AZT is involved in a number of activities in which non-azidylated ddNs are not. First, it has been shown that AZT administered to animals is converted by the liver to 3′-amino-3′-deoxythymidine, itself highly toxic to cells [17]. Second, exposure of cells to AZT leads to extensive alterations to mtDNA; a high proportion of guanine residues become hydroxylated at the 5 position [18]. Third, AZT stimulates mitochondrial proliferation in the Friend cell [6, 12]. (We have not observed ddC to have this effect in either the Friend cell or the PC12 cell.)‖ Fourth, it has been reported that AZT inhibits the transcription of the globin gene

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§ Abbreviations: ddN, dideoxynucleoside; AZT, 3′-azido-3′-deoxythymidine; ddC, dideoxycytidine; ddI, dideoxyinosine; d4T, 3′-deoxythymidin-2′-ene; KRP, Krebs–Ringer phosphate solution; NAO, *N*-nonyl acridine orange; and TCA, trichloroacetic acid.

‖ Keilbaugh SA, Hobbs GA and Simpson MV, manuscript in preparation.

[19]. Finally, although both sets of drugs can inhibit mtDNA replication via DNA polymerase γ as mentioned earlier, the mechanism by which AZT triphosphate acts on this enzyme differs from that of the triphosphates of non-azidylated ddNs, namely, competitive or partial competitive inhibition in the case of AZT triphosphate and chain termination [8, 9] in the case of the others.

We report here yet another mode of action specific to AZT. Prior to the commencement of the present study, although strong evidence existed that many ddNs, including AZT, could inhibit mtDNA replication, we had not yet substantiated our hypothesis [3] that such inhibition would result in the impairment of oxidative phosphorylation. (While such an effect had indeed been shown indirectly for ddC [10], this analog, as was pointed out previously, acts on DNA polymerase γ by a mechanism different from that of AZT.) Nor had we yet demonstrated a cause-and-effect relationship between the AZT inhibition of mtDNA replication (or its consequence, an impairment of oxidative phosphorylation) on the one hand and an inhibition of cell growth on the other. Thus, the possibility had not been eliminated that AZT was exerting some general cytotoxic effect on the cell, which resulted in an inhibition of cell growth, and this, in turn, was leading to an inhibition of mtDNA replication. Therefore, we were also concerned with the question of whether an appropriate temporal relationship exists among three events, namely, the inhibition of mtDNA replication, its metabolic consequences, and the decrease in cell growth, specifically, whether these events occur in this sequence. These experiments, done with the Friend cell, necessitated using increasingly short time intervals after addition of AZT, and it was this that brought to light the new activity of AZT. We noticed that the beginning of the AZT-induced inhibitory effect on cell growth occurred at a relatively short time after AZT addition to the medium, a period of time too short to account for the effect to have been brought about by an inhibition of mtDNA replication. This observation led to studies of the early metabolic events that occur upon exposure of the cells to AZT, and these results are reported here. We also describe some protein synthesis studies bearing on the mechanism of the proliferation of mitochondria. Finally, we describe results showing that aminothymidine could not be responsible for these or other results of our studies on the Friend cell.

MATERIALS AND METHODS

Growth of cells. Cells were grown in medium A (RPMI 1640, penicillin, streptomycin, 10% dialyzed bovine calf serum and 5% dialyzed fetal bovine serum) or medium B (RPMI 1640, penicillin, streptomycin, and 15% dialyzed bovine calf serum), as designated in the legends to the figures. At each time point, both in the cell growth experiments or in cases in which samples were removed from the culture for further analysis, cells were counted using a Coulter counter. In the case of the latter samples, the culture was split at every time point (see legend to Fig. 2), and samples were taken for the various analyses. All such samples were stored on ice unless otherwise noted.

Measurement of O_2 uptake. For studies of O_2 uptake, a 50 mL sample of the split cell culture was centrifuged at 2000 *g* for 10 min at 4° to sediment the cells. The supernate was used for lactate analysis. The pellet was resuspended in 0.5 mL of cold KRP [20] and kept on ice. The cell count was determined, and a sample containing 2×10^7 cells was transferred to an Eppendorf tube and set in ice. Disparate volumes of all such samples were equalized by addition of cold KRP. The cell suspension was then introduced into a 3 mL volume of KRP preequilibrated to 37° in a polarographic apparatus (Yellow Springs Instrument Co., Yellow Springs, OH), O_2 consumption being followed with the Clarke electrode. Washing the electrode with 95% ethanol between samples almost invariably reduced blank values to nearly zero.

For the O_2 uptake analyses related to the uncoupling and respiratory control experiments (Figs. 5 and 6), mitochondria isolated from the livers of Sprague–Dawley rats were used. Determination of their O_2 uptake capacity was performed according to the respiratory control method of Estabrook [21] with the exceptions that the oxidizable substrate was α-ketoglutarate* (added in the form of potassium glutamate), triethanolamine was replaced by Tris, and O_2 measurements were made polarographically using the Clarke electrode, as described earlier.

Measurement of lactate/cell. For lactate determination, 100 μL of the supernate from the cell centrifugation described earlier was transferred to an Eppendorf tube containing 200 μL of cold 10% TCA and permitted to stand in ice for 5 min. The precipitate was removed by centrifugation, and 200 mL of supernate was transferred to an Eppendorf tube and stored at −80° pending assay. The assay, performed using the Sigma lactic acid kit (826-A), and following the manufacturer's instructions, depends upon the lactic dehydrogenase catalyzed reduction of NAD to NADH and the spectrophotometric determination (340 nm) of the latter. In our case, 28 μL was transferred from the thawed sample to 800 mL of the Sigma assay mixture. We corrected for the small blank value given by the unincubated culture medium.

Measurement of ATP. For measurement of cellular ATP content (ATP/cell), ATP was determined by bioluminescence using the Sigma luciferin/luciferase kit and following the accompanying instructions. After cell counting, a 200 μL sample of each culture medium was transferred rapidly to an Eppendorf tube on ice containing 200 μL of the Sigma somatic cell releasing agent, after which the suspension was frozen at −80° pending assay, usually in about 10 min. For assay, the "released" cell suspension was thawed, and 100 μL was mixed rapidly with 100 μL of the 10-fold diluted Sigma assay mixture in a disposable cuvette that had been placed previously in the Perkin Elmer MPF-44a spectrofluorimeter, which served as a highly sensitive and high resolution luminometer; incident light was turned off, and the

* Penefsky H, personal communication. Cited with permission.

photometric mode used (panel AC/DC switch) was DC. Bioluminescence was measured at 562 nm on the initial plateau (not the initial flash) of the curve tracing, and particular care was exercised to treat all samples identically with respect to the length of the stirring time and the time of initiation of the recording. Arbitrary pen deflection units were quantified using an ATP standard curve. The experiment was done from the start with three independent AZT flasks and three independent control flasks.

For determination of ATP synthesis in mitochondria isolated from Friend cells, the cells were incubated for 72 hr with 10 μM AZT, the mitochondria were isolated as described previously [6], and measurement of ATP formation was carried out according to Nielsen and Lehninger [22].

Measurement of mitochondria/cell. Mitochondrial content could be conveniently measured by taking advantage of the specific binding of the fluorescent probe NAO to mitochondrial membranes [23, 24]. We have found this method to yield results that are in fairly good agreement with those obtained by determining the amount of protein in mitochondrial pellets or by counting mitochondria by electron microscopy [6]. A 6.5-mL sample was withdrawn from the cell culture at the designated time point, 3 mL of which was kept in ice as a zero time sample and 3 mL was made to 5 μM NAO and incubated for 15 min at 37°. The incubated sample was then chilled on ice and after adding an equal amount of NAO to the zero time sample, both 3-mL samples were centrifuged at 2000 *g* for 10 min at 4°. The pelleted cells were washed three times with PBS solution (0.138 M NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.71 mM KH_2PO_4, pH 7.4) and resuspended in 3 mL PBS. Fluorescence was determined in a Perkin Elmer MPF-44a spectrofluorimeter. Excitation was set at 488 nm and emission measured at 525 nm.

Measurement of protein synthesis. After growth of Friend cells for 3 days with and without 5 μM AZT, the cells were pelleted (500 *g* 10 min, Beckman TJ-6 rotor) under aseptic conditions, washed once with sterile PBS, and resuspended in the original volume of medium C (methionine-free medium B supplemented with 1.8% DMSO). After preincubation for 10 min with 100 μg/mL of chloramphenicol (to inhibit mitochondrial protein synthesis) under the usual cell growth conditions, the labeled amino acid mixture was added (1 μCi/mL [^{35}S]methionine/[^{35}S]cysteine mixture, New England Nuclear), and the incubation was continued for 2 hr. A flask was kept on ice for a zero time sample. At the end of the incubation, the flasks were chilled and all further steps were carried out in the cold. The cells were collected by centrifugation and were washed once with 20 mL of sterile PBS. The final pellet was resuspended in hypotonic solution (10 mM Tris, pH 7.1, 10 mM KCl, 150 μM $MgCl_2$) for 10–15 min after which the cell suspension was disrupted with a hand-driven Potter–Elvehjem homogenizer with a tight-fitting Teflon pestle, and immediately made to 0.3 M sucrose. Mitochondria and post-mitochondrial supernate were isolated essentially as originally described by Schneider and Hogeboom [25] except that the sucrose concentration was 0.3 M instead of 0.25 M. Mitochondrial protein and the protein of the post-mitochondrial supernate were precipitated by adding a sample of each to a TCA solution (10% TCA, 10 mM methionine, 10 mM cysteine, 14% β-mercaptoethanol). After standing for at least 15 min in the cold, the TCA precipitates were collected onto nitrocellulose filters that were washed with TCA and then isopropanol; the filters were dried, and counted, and specific activities were calculated.

Detection of 3'-amino-3'-deoxythymidine. Friend cells were incubated for 72 hr under the usual conditions in 10 mL of medium B plus 1.8% DMSO; the AZT concentration was 5.0 μM to which were added 6000 cpm of [^{3}H]AZT (11 Ci/mmol). At 5 days, the medium was centrifuged to remove the cells, and the supernate was deproteinized with TCA, brought to pH 8.0 with 1 M Tris buffer, pH 8.0, and subjected to HPLC reverse phase chromatography using the Perkin Elmer Isopure LC system. Analysis was done with an analytical Zorbax ODS-2 column [26], 4.6 mm i.d. × 25 cm, protected by a Waters C18 Sep-Pac unit. The Sep-Pac was wetted with two successive 1 mL portions of methanol, hydrated and equilibrated with water (four successive 1 mL portions). A 10 μL sample of the cell culture medium was loaded onto the Sep-Pac, which was then washed twice with two 1 mL volumes of water. The water was displaced with 0.7 mL of methanol and elution was with 1 mL methanol. It had been established previously that when an authentic sample of aminothymidine is applied to a Sep-Pac unit and eluted under these conditions, virtually 100% of the sample is recovered. The eluate was dried under vacuum and was redissolved in 250 μL of polar buffer (10 μM K_2HPO_4, pH 3.0: methanol, 19:1), filtered through a Millipore Millex GV4 0.22 μm filter unit, and injected into the chromatography apparatus, the Zorbex column having been preequilibrated with polar buffer for at least 10 min. The flow rate was 1.0 mL/min with the following program: polar buffer for 5 min; 10 μM KH_2PO_4, pH 3.0: methanol, 1:3, slope = 1, for 30 min; methanol for 10 min, slope = 0. Fractions were collected one per minute, an aliquot was spotted on GF/C filters, and the radioactivity was determined by scintillation counting. The retention positions of the AZT and aminothymidine markers were determined by measuring the absorbance at 260 nm of the fractions from the control flask. The aminothymidine peak emerged at 9.5 min and the AZT peak at 30 min, both reproducibly.

RESULTS

Effect of long-term incubation of Friend cells with AZT on cell growth and oxidative phosphorylation. DMSO-induced Friend cells were incubated for 5 days with 5 and 25 μM AZT, samples of the medium were removed daily, and the cells were counted. The results (Fig. 1) showed marked inhibition of cell growth even at the lower AZT concentration. These data have been published previously [6] in separate figures, but are reproduced here in a single graph to

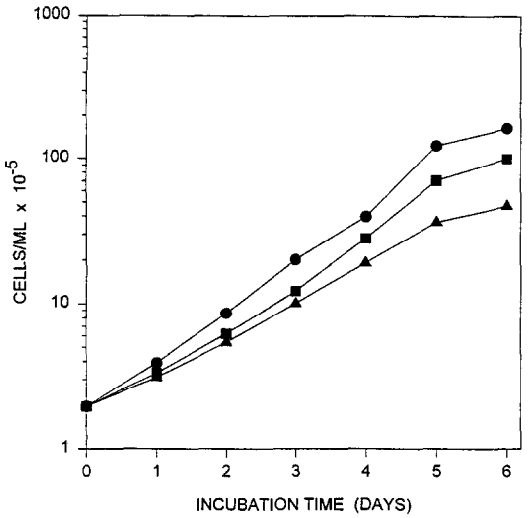

Fig. 1. Effect of long-term incubation with AZT on growth of Friend cells. Uninduced Friend murine erythroleukemic cells were grown in 25-mL canted neck flasks in medium A. Cells were seeded at 1.9×10^5 cells/mL into Falcon round bottom polypropylene tubes containing 5 mL medium A, 1.8% in DMSO, with and without AZT. The loosely capped tubes were drum rotated (6 rpm, 80° inclination) in humidified air/5% CO_2 at 37°. Each of the two passages was at $1–2 \times 10^6$ cells/mL. Cell density was measured by Coulter counting. The results are representative of many identical experiments. Key: (●——●) control (no AZT); (■——■) 5 μM AZT; and (▲——▲) 25 μM AZT. This material was adapted from results shown in Ref. 6.

supply the context for the next three experiments to be discussed.

We have also shown previously that DMSO-induced Friend cells isolated after a 60 hr incubation with AZT become seriously defective in mtDNA replication [6]. To substantiate our hypothesis [3] that such a defect would lead to an impairment of oxidative phosphorylation, we isolated control and AZT-incubated DMSO-induced cells (1 μM AZT) daily for 5 days and analyzed them for their rates of oxygen uptake, lactic acid production, and ATP content.

The results (Fig. 2) show clearly that a disturbance in these parameters was occurring. The fact that this experiment was done on as complex a system as the whole cell, together with the fact that mitochondria proliferate in the presence of AZT [6], makes the day-to-day results on the three parameters difficult to correlate with one another. Nevertheless, the steep rise in lactate production, in agreement with the effect of ddC on Molt F cells [10], suggests an impairment of oxidative phosphorylation. The increase in O_2 uptake supports this view and further suggests that the impairment results from an uncoupling of phosphorylation from terminal electron transport, at least in part. The wide variation in ATP levels is not surprising given the increased rate of glycolysis and the fact that ATP is involved in so many cellular reactions.

Table 1. Effect of growth of Friend cells in 10 μM AZT on oxidative phosphorylation in mitochondria isolated from such cells

AZT	ATP formed* (cpm [^{32}P]ATP)	ATP formed (mean cpm)	Inhibition (%)
None	3880		
	5270	4260	
	3630		
10 μM	1450		
	2740	2320	45.5
	2760		

* Mitochondria were isolated from DMSO-induced Friend cells after a 72-hr incubation of the cells with 10 μM AZT. Their ability to synthesize ATP was measured by the [^{32}P]orthophosphate method of Nielsen and Lehninger [22]. Each datum is derived from cells grown in a separate flask and treated separately. Each reaction mixture contained 130 μg of mitochondrial protein.

Conclusive evidence, however, of the impairment of oxidative phosphorylation would best come from the direct demonstration that the mitochondria of AZT-grown cells are defective in ATP synthesis. To obtain such evidence, cells were incubated with 10 μM AZT for 72 hr under incubation conditions identical to those of the previous experiment, and mitochondria were then isolated. The ability of these mitochondria to synthesize ATP was then tested directly using the classical [^{32}P]orthophosphate method of Nielsen and Lehninger [22]. The results showed (Table 1) almost a 50% inhibition of ATP synthesis in the mitochondria from the cells grown in AZT.

Effect of short-term incubation with AZT on Friend cell growth. Upon examination of the curves shown in Fig. 2, we were struck by the fact that the metabolic changes following AZT addition had already occurred by day 1. This was unexpected since the results of Chen and Cheng with ddC on the molt F cell [10] and our own results with ddC on the PC12 cell [5] point to a delayed effect of several days duration. Furthermore, on re-examination of the day 1 points in the 5 μM curves in Fig. 1, we realized that there was already a suggestion of a depression of cell growth in the AZT growth curve. Such a depression is considerably more evident in the 25 μM curve, albeit at an AZT concentration unpharmacologically high.

These results led us to examine the growth curve at shorter time intervals, again at 5 μM AZT. They show (Fig. 3) that the inhibition of cell proliferation began within 3 hr after addition of AZT. The results of a similar experiment (done only once and not shown) in which the concentration of AZT was increased to 10 μM are in agreement with these results except that the extent of inhibition was approximately twice as high.

Effect of short-term incubation of Friend cells with AZT on O_2 uptake, lactate production, ATP content, and mitochondrial proliferation. The preceding results suggested that AZT was exerting an immediate effect on some metabolic step crucial to

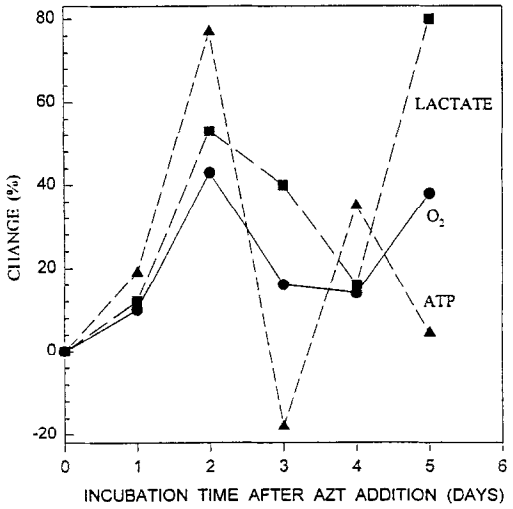

Fig. 2. Effect of long-term incubation of Friend cells with AZT on O_2 uptake, lactate production and ATP level. Uninduced cells were grown as described in the legend to Fig. 1. They were seeded into 150 mL of medium A, 1.8% in DMSO, at 5×10^5 cells/mL and incubated with and without AZT (1 μM) under the conditions described in the legend to Fig. 1. Cell suspensions were split daily, the removed cell suspension (50 mL) being used for analysis as described in the text and the remainder reincubated after the cell concentrations of both control and AZT samples were adjusted to 5×10^5 cells/mL, and the AZT concentration to 1 μM, the volumes being adjusted to 150 mL. Control values at various incubation times follow. O_2 uptake in μmol $O_2/10^7$ cells/10 min: at 1 day, 0.385; at 2 days, 0.375; at 3 days, 0.412; at 4 days, 0.440; at 5 days, 0.311. Lactate content of medium in μmol/10^7 cells: at 1 day, 108.4; at 2 days, 58.9; at 3 days, 68.4; at 4 days, 76.0; at 5 days, 48.9. Cellular ATP content in ng ATP/10^4 cells: at 1 day, 0.181; at 2 days, 0.244; at 3 days, 0.351; at 4 days, 0.147; at 5 days, 0.162. The plot represents the percent difference between AZT and control samples at each time point. This experiment was repeated twice. Key: (●——● O_2 uptake; (■——■) lactate production; and (▲——▲) ATP per cell.

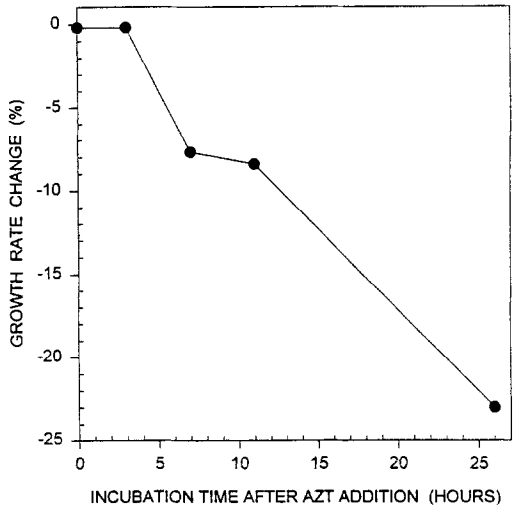

Fig. 3. Effect of short-term incubation with 5 μM AZT on Friend cell growth. Conditions were as described in the legend to Fig. 4. This experiment was performed twice at this AZT concentration; see text for effect of higher concentration.

cell growth. A number of considerations including the immediacy of the AZT effect suggested that the target might be cytoplasmic rather than nuclear (e.g. nDNA replication, mRNA synthesis). We decided to first examine oxidative phosphorylation, a complex process interlinked with many other cellular events and whose impairment would be likely to lead to a rapid inhibition of cell growth. Thus, we harvested AZT-grown and control cells at 3, 7, 11, and 26 hr and measured their O_2 uptake, their lactate and ATP levels, and their mitochondrial content.

The event that was most notable because of the rapidity with which it occurred was a dramatic drop in cellular ATP levels at the first time point, a drop of 30% within 3 hr of exposure to AZT (Fig. 4). On the other hand, O_2 uptake increased slightly (about 6%) during this period. The lactate concentration rose slightly during this period but began to undergo a rapid rise at 7 hr; at 11 hr, lactate had increased

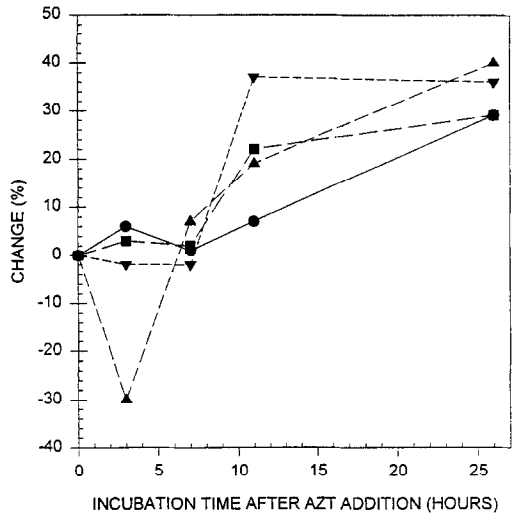

Fig. 4. Effect of short-term incubation of Friend cells with 5 μM AZT on O_2 uptake, lactate production, ATP level, and mitochondria per cell. Cells were seeded at 1.8×10^5 cells/mL into 500 mL of medium B containing 1.8% DMSO and incubated for 3 days in a spinner flask in humidified air/5% CO_2 at 37°. After dilution with medium B to about 8×10^5 cells/mL, the suspension was divided into two moieties, one of which was made to 5 μM AZT, and the incubation continued in two 500-mL spinner flasks. Samples were withdrawn at 3, 7, 11 and 26 hr and treated as described in Materials and Methods. To compensate for the increased error inherent in measuring the smaller changes that occur in short-term incubation experiments, we used the mean values from four experiments to plot these curves. Key: (●——●) O_2 uptake; (■——■) lactate production; (▲——▲) ATP per cell; and (▼——▼) mitochondria/cell.

by about 20% and continued to increase after that but at a slower rate. Such data obtained in a system less complicated than the whole cell would suggest an uncoupling of oxidative phosphorylation, but this conclusion cannot validly be drawn from these data alone. The 6-hr time point was witness to other events as well. Cellular ATP returned to its initial concentration at 6 hr but rose rapidly thereafter to 19% above the control at 11 hr and continued to increase rather steeply up to 26 hr, the termination of the experiment. The results in Fig. 2, however, suggest that the rise in cellular ATP continued for another 24 hr. The number of mitochondria per cell (NAO curve) also began to increase at 6 hr and appeared to level off at about a 37% increase over the control at 11 hr. A previous study [6] using a single time point of 60 hr in which three independent methods were employed to determine mtDNA/cell (electron microscopy, protein content of mitochondrial pellets, and NAO) shows an increase of 60% for the average of the three methods and 58% for the NAO alone.

It is clear from these results that AZT brought about rapid alterations in cellular metabolism beginning at least as early as 3 hr in the case of ATP and at 6 hr in the case of the other parameters measured. The limitations of our currently available data, however, do not permit a definitive correlation of the chronological relationships of these four variables to one another, given their interdependence as well as the complexity of a whole cell system. For example, the change in the rate of utilization of ATP in the AZT-grown cells is unknown as is the rate of synthesis by the proliferating mitochondria, or the extent to which glycolysis replaces oxidative phosphorylation in the synthesis of ATP. Also, the presence of DMSO could further complicate any attempt at such correlation because of its tendency to induce cell synchronization [27, 28]. The results do make clear, however, that oxidative phosphorylation was deleteriously affected in some manner.

Is AZT an uncoupler of oxidative phosphorylation? There are many possible ways in which AZT might inhibit oxidative phosphorylation. AZT could interact with mitochondria directly; for example, it might cause uncoupling, it could inhibit one of the steps in terminal electron transport, or it could inhibit the step in which phosphorylation of ADP occurs. Alternatively, AZT could act indirectly; for example, it could inhibit the adenylate kinase reaction that furnishes ADP (the sole phosphate acceptor) to the oxidative phosphorylation system, it could inhibit the respiratory control system (by inhibition of adenylate kinase or in some other way), it might interfere with a step in fatty acid breakdown, or it might inhibit the adenylate or the acylcarnitine transport system.

To test the uncoupling possibility, we made use of the classical respiratory control system, except that α-ketoglutarate (added as glutamate) was used as the oxidizable substrate in place of glutamate. We asked whether, in a properly operating system, the addition of AZT would result in an increase in O_2 uptake. Because of the amount of mitochondria required, rat liver was used as the source. The results (Fig. 5) of the addition of ADP to the mitochondrial

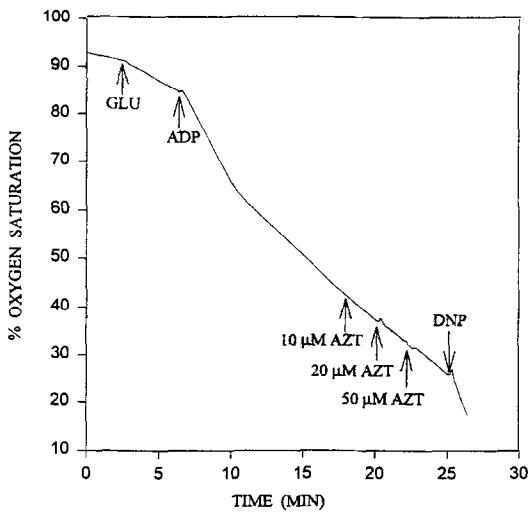

Fig. 5. Effect of AZT on O_2 uptake by isolated mitochondria. Rat liver mitochondria were suspended in KRP, and their O_2 uptake in the presence of the indicated substances was measured polarographically. The reaction mixture was equilibrated with air, the equilibration point being arbitrarily set at 100% O_2 saturation, a value equivalent to 1440 μatoms of O_2. This experiment was performed twice. The arrows indicate the time of addition of the indicated substances whose final concentrations were: potassium glutamate, 10 mM; ADP, 0.3 mM; AZT at 18 min, 10 μM; AZT at 20 min, 20 μM; AZT at 22 min, 50 μM; DNP (2,4-dinitrophenol), 20 μM.

preparation used in this experiment showed that respiratory control is operating properly; the respiratory control index was about 3. It is well-established that uncoupling of oxidative phosphorylation results in a sharp increase in O_2 uptake, and this is shown here by the addition of 2,4-dinitrophenol as a control. The system is thus well coupled and operating properly. The results showed no effect whatsoever upon addition of AZT; no uncoupling effect could be seen, even at AZT concentrations as high as 50 μM. Thus, it does not appear that AZT *per se* is an uncoupler of oxidative phosphorylation.

Does AZT interfere with respiratory control? In this experiment, we used the same system as was used for Fig. 5. Here, we asked whether the presence of AZT would inhibit the stimulatory effect of ADP on O_2 uptake, i.e. respiratory control. The results demonstrated (Fig. 6) that when ADP was added in the absence of AZT (at 9 min), stimulation of O_2 uptake occurred; this stimulation was not interfered with when ADP was added in the presence of 50 μM AZT. Thus, respiratory control is not impaired by AZT *per se*.

Effect on cellular protein metabolism of long- and short-term incubation with AZT. As observed previously in our and other laboratories, prolonged exposure to AZT induces mitochondrial proliferation, both in cell culture and *in vivo* (in muscle cells) [6, 12]. This would require increased activity of the mitochondrial protein synthesis system which

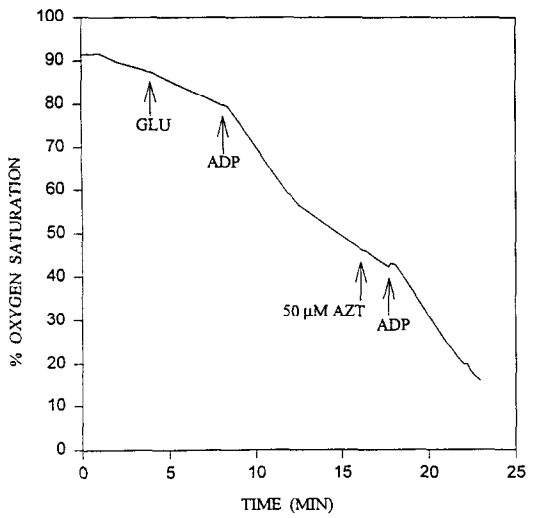

Fig. 6. Effect of AZT on respiratory control in isolated mitochondria. Conditions were the same as described in the legend to Fig. 5. The experiment was performed twice.

needs to produce only 13 proteins, as well as of the cytoplasmic system which would need to produce a far greater number. It was therefore of interest to ask what changes were, in fact, occurring in cytoplasmic protein synthesis. To answer this question, Friend cells were incubated with and without 5 μM AZT for 72 hr, washed, and reincubated for a short period with labeled amino acids ([^{35}S]methionine/[^{35}S]cysteine) in the presence of chloramphenicol, a powerful inhibitor of mitochondrial protein synthesis. Cytoplasmic and mitochondrial fractions were then isolated, the protein was precipitated and washed, and the radioactivity was measured. The results (Table 2) show that a small but reproducible decrease, averaging about 13%, in overall cytoplasmic protein synthesis took place. In contrast, a marked increase was observed (averaging about 28%) in the labeling of protein isolated from the mitochondria, indicating an increased rate of synthesis of those proteins made in the cytoplasm and imported into mitochondria.

Inasmuch as we had obtained evidence that mitochondrial proliferation commenced at about 6 hr after exposure of the cell to AZT (Fig. 4), we were interested in seeing whether these results would be reflected in changes in the rates of synthesis of cytoplasmic proteins and of imported mitochondrial proteins. In an experiment of identical design to that of the previous experiment in Table 2, little or no change (−2.1%) could be observed in overall cytoplasmic protein synthesis (Table 2, experiments 2a and 2b). In contrast, a 66% increase in the synthesis of imported mitochondrial protein occurred. Thus, these results are consistent with the finding that AZT induces mitochondrial proliferation and will be further discussed later.

Do Friend cells convert AZT to aminothymidine? It has been observed that *in vivo*, AZT can be converted to aminothymidine, itself even more toxic than AZT to cell growth in culture [24]. While the liver is thought to be the only organ that brings about this conversion, we nevertheless thought it important to show that it was not occurring in the Friend cell, i.e. that our results were not being distorted by the presence of this AZT catabolite. After incubation of cells with 5 μM [^{3}H]AZT for 72 hr, the culture medium was analyzed for labeled aminothymidine by HPLC chromatography. The peak value in the AZT region was 2450 cpm, but only a trace of radioactivity above background was found in the aminothymidine region (Fig. 7).

DISCUSSION

In the work presented here on the mechanism of

Table 2. Effect of long- and short-term incubation of Friend cells with AZT on the synthesis of imported mitochondrial proteins and cytosolic proteins

Conditions			Imported mitochondrial protein*		Cytosolic protein†	
Expt.	Incubation time	[AZT]	Sp. act. (cpm/mg)	Change (%)	Sp. act. (cpm/mg)	Change (%)
1a‡	72 hr	None	840		1770	
		5 μM	1010	+21	1520	−14
1b	72 hr	None	2500		3680	
		5 μM	3350	+34	3290	−11
2a	7 hr	None	3260		4160	
		5 μM	5740	+76	3900	−6.1
2b	7 hr	None	3980		3930	
		5 μM	6150	+55	4010	+2.0

* Measured by incubating cells with [^{35}S]methionine/[^{35}S]cysteine in the presence of 100 μg/mL chloramphenicol.

† Post-mitochondrial supernatant protein.

‡ Experiments 1a and 1b were performed under similar conditions except that different amounts of labeled precursor were used.

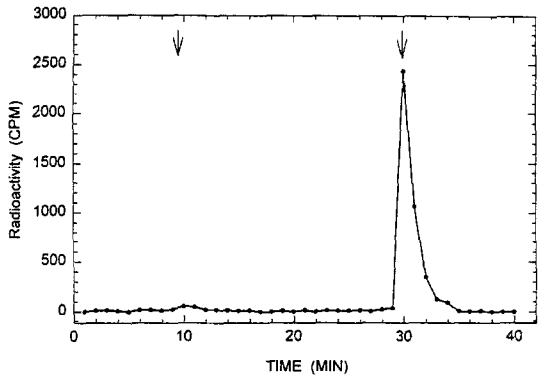

Fig. 7. HPLC chromatogram of cell culture medium in which Friend cells were incubated for 5 hr with [^{3}H]AZT. A description of the procedure is given in the text. The arrows represent the positions of unlabeled aminothymidine (left) and AZT (right) markers run in a parallel experiment. Blank values were subtracted in the case of all fractions except in the case of the fractions containing labeled AZT (28–35) where this was not possible. They were obtained by running two additional parallel experiments in which labeled AZT was incubated, one in water, the other in cell-free incubation medium. The blank values were negligible (except in the fractions mentioned earlier), close or equal to radioactivity background.

AZT toxicity, particularly that toward bone marrow, we have chosen the Friend murine erythroleukemic cell for our studies. This cell line has long been used as a model for a part of the bone marrow erythropoietic system. It is now well established that AZT and other dideoxynucleosides inhibit mtDNA replication, and our earliest work on this problem demonstrating such an inhibition led us to predict that it would lead to an impairment of oxidative phosphorylation [3]. The experiments described here on the effect of a 5-day incubation of Friend cells with AZT on O_2 uptake and lactic acid formation provide strong but indirect evidence of such an effect. Moreover, we have obtained for the first time *direct* evidence for the impairment of oxidative phosphorylation by a ddN; mitochondria isolated from cells grown in the presence of pharmacological levels of AZT (5 μM) for 5 days and tested for their ability to carry out oxidative phosphorylation showed a marked decrease in ability to synthesize ATP.

These and other *in vitro* studies with AZT and ddC as well as *in vivo* AZT studies on laboratory animals and AIDS patients have suggested that the effects of ddNs on mitochondrial structure and metabolism are delayed, consistent with DNA polymerase γ being the target of the drug. In the course of some recent short-term incubation experiments, however, we noted that the beginning of the effect on cell growth seemed to occur shortly after the initiation of exposure to AZT, suggesting that, in addition to these previously observed *in vitro* and *in vivo* effects, an immediate effect of AZT was occurring in our system. Further studies of this phenomenon in which the frequency of sampling the medium was in hours rather than days confirmed this early effect of AZT on cell growth and also showed early changes in O_2 uptake, lactate synthesis, ATP level, and number of mitochondria per cell. Some of these changes, particularly that of ATP level, were observable as early as 3 hr after exposure to AZT and, judging from the precipitous decline of the ATP/cell curve between 0 and 3 hr (Fig. 4), may have begun earlier than that. The 3 hr time interval, equivalent to only 7% of the doubling time of the AZT-treated cells, is far too short a period of time to account for the effect brought about by an inhibition of mtDNA replication. An effect such as the latter depends, as we have shown previously [6], upon succeeding generations of cells running out of functional mtDNA, a molecule that is present in mitochondria in multiple copies. The results are thus in accord with the view that AZT must be exerting an immediate direct effect on some step in metabolism, an effect that is manifested by an impairment of oxidative phosphorylation. Since we have thus far shown this phenomenon to exist only in our *in vitro* system, it would be of importance to investigate whether it also occurs *in vivo*, particularly in AZT-treated AIDS patients.

We demonstrated here, using isolated mitochondria, that AZT neither uncouples oxidative phosphorylation nor interferes with the process of respiratory control of oxidative phosphorylation. Moreover, it did not inhibit the synthesis of ADP (the sole phosphate acceptor in oxidative phosphorylation) via the enzyme responsible for this synthesis, mitochondrial adenylate kinase [16]. We are therefore left with the conclusion that the process of oxidative phosphorylation itself is not the *direct* target of AZT. Rather, we propose that, just as exposure of cells to AZT and ddC immediately and directly inhibits mtDNA replication which, in turn, given a sufficiently long exposure time, leads to the inhibition of oxidative phosphorylation, short-term exposure to AZT influences some unknown metabolic step and this, in turn, immediately leads to an impairment of oxidative phosphorylation. A possible approach to the identification of this target might come from preliminary experiments which suggest that AZT has a predilection for binding to enzymes that contain a paired ATP/ADP binding site [16]. Other possibilities could include the involvement of some of the targets of AZT mentioned at the beginning of this article as well as a yet unknown target.

The immediate and delayed effects of AZT are probably independent of each other. It is not likely that the rapid AZT effect on oxidative phosphorylation can be caused by the inhibition of mtDNA replication and the subsequent depletion of mtDNA. The depletion of mtDNA requires the passage of a number of generations particularly since each mitochondrion contains several genomes, and only the one involved in replication would be affected by AZT. The inhibition of ATP synthesis, however, begins in less than 0.07 generations. It is likely, however, that the early effects of AZT do not cease after a short time but continue with time so that they would add to the delayed effects of AZT.

We have studied further the observation made in our [6] and other [12] laboratories that AZT induces

mitochondrial proliferation. In long-term (5-day) incubations with AZT, the increased rate of synthesis of those mitochondrial proteins synthesized in the cytoplasm and imported into mitochondria is accompanied by a decreased rate of synthesis of cytosolic proteins, suggesting that the former is taking place at the expense of the latter, but further experiments are required to substantiate the occurrence of such a "redirection" of protein synthesis. The increased rate of synthesis of imported proteins is also seen after short-term (7 hr) incubation (with, however, at most only a small decrease in cytosolic protein synthesis) and strengthens the view of the existence of an immediate AZT effect. While it is probable that the proliferation of mitochondria is an indirect effect of ATP depletion, it is not ruled out that it is caused by the interaction of AZT with yet another cellular target, thus far unidentified.

We also show that the various effects of AZT on the Friend cell described both here and in previous publications from this laboratory cannot be ascribed to aminothymidine. The latter is a catabolic product of AZT shown to be produced by the liver [17].

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