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Effect of 2′,3′-Dideoxycytidine On Oxidative Phosphorylation in the PC12 Cell, a Neuronal Model

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ABSTRACT. Peripheral neuropathy induced by 2′,3′-dideoxycytidine (ddC) could result from the previously shown inhibition of mtDNA replication by the action of ddC on the mitochondrial enzyme DNA polymerase γ. Such inhibition would be expected to impair oxidative phosphorylation, and this was demonstrated in the present study for the PC12 cell, a model of a peripheral neuron. The dramatic rise in lactate formation upon exposure of the cell to ddC indicated that increased glycolysis was needed to produce ATP. A concomitant rise in O_2 uptake indicated that oxidative phosphorylation had become uncoupled. When tested in a standard respiratory control system (isolated rat liver mitochondria), however, we found ddC not to be an uncoupler. Rather, the uncoupling most likely resulted from the failure of synthesis of one or more mitochondrial gene products necessary for oxidative phosphorylation. We also observed an important distinction between the manner in which ddC and 3′-azido-3′-deoxythymidine (AZT) act. ddC-exerted inhibition of oxidative phosphorylation was delayed for several days. This is consistent with the inhibition occurring indirectly, most likely as a result of the prior destruction of the mitochondrial genome, which encodes many of the components of the oxidative phosphorylation system. In contrast, we have shown previously that although AZT also impairs replication of the mitochondrial genome (in the Friend murine erythroleukemic cell), it also attacks directly an additional primary target leading to impairment of oxidative phosphorylation; its initial inhibition of this process is immediate, not occurring via inhibition of mitochondrial DNA replication. BIOCHEM PHARMACOL 53;10: 1485–1492, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. dideoxynucleosides; dideoxynucleoside toxicity; dideoxycytidine toxicity; ddC toxicity; oxidative phosphorylation; mitochondrial DNA

Evidence from our [1–5] and other laboratories (citations in Refs. 2 and 5) has led to the recognition that an important target of AZT§ in the mammalian (including the human) cell is the mitochondrion and, more specifically, the mitochondrial enzyme DNA polymerase γ, leading to severe depletion of the mitochondrial genome. The evidence also includes a resulting deleterious effect on oxidative phosphorylation and on the structure of the mitochondrial inner membrane, indicating that these phenomena, at least in part, are likely implicated in the more serious toxic side-effects of AZT, namely bone marrow suppression and skeletal and cardiac myopathy.

Recently, using the Friend murine erythroleukemic cell (a cell culture line widely used as a model of the bone marrow erythropoietic system), we have detected an additional, apparently non-mtDNA-associated, effect of AZT on cellular metabolism [5]. While the phenomenon manifests itself as an inhibition of both oxidative phosphorylation and cell growth, the effect is virtually immediate, thus differing from the indirect delayed effects on metabolism expected from a mechanism based on suppression of mtDNA replication.

Evidence exists that the mechanism of the toxic side-effects exerted by non-azidylated ddNs (primarily ddC/ddI/d4T-associated peripheral neuropathy) also involves inhibition of mtDNA replication. Treatment of Molt-4F cells (a non-neuronal cell line) with ddC results in depletion of mtDNA and concomitant impairment of oxidative phosphorylation [6]. Subsequent studies on the PC12 cell (widely employed as a model of a neuronal cell) showed that treatment of the cell with ddC inhibits cell proliferation and mtDNA replication [7, 8] and leads to depletion of mtDNA [9]. In this report, we take up the question whether a neuronal cell (our model, the PC12 cell) will also suffer impairment of oxidative phosphorylation in response to ddC treatment. Moreover, we also ask whether ddC, like AZT, might also exert an immediate effect on oxidative phosphorylation.

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§ *Abbreviations:* AZT, 3′-azido-3′-deoxythymidine; HIV-1, human immunodeficiency virus; ddN, dideoxynucleoside; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; d4T, 3′-deoxythymidin-2′-ene; NGF, nerve growth factor; and mtDNA, mitochondrial DNA.

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MATERIALS AND METHODS

Cell Culture

GS-*ras*-1 or GS-*ras*-2 cells, c-Ha-*ras* transformants of the PC12 cell [10], were used in these experiments. They were more convenient to use for our purposes than the PC12 cell itself, since dexamethasone instead of NGF can be used for the induction of differentiation, which proceeds much more rapidly than with NGF. The GS-*ras*-2 transformant cell proved easier to use than the GS-*ras*-1 when differentiated cells were required, since the GS-*ras*-2 cells clump less, permitting more efficient neurite outgrowth. This cell became available to us during the course of these experiments; in pilot experiments, its response to ddC was similar to that of the GS-*ras*-1 cells.

The cells were maintained in tissue culture dishes in Dulbecco's modified Eagle's medium (Gibco catalogue No. 320-1965PJ) fortified with horse serum and fetal bovine serum (90:10:5) and containing 1000 units of penicillin G sodium and 1000 μg/mL streptomycin sulfate. Cultures (maximum cell density 2.5×10^5 to 1.0×10^6 cells/mL) were passaged every 5 days into 105 mL of fresh medium, from which 20-mL volumes were used to seed five 100-mm tissue culture plates. All cell culture incubations were at 37° in 10% CO_2 and in 98% humidity. Dexamethasone was used at a concentration of 0.075 μM, and was added 1 day after seeding the sample.

Cultures for the lactate studies in proliferating cells were seeded into multiwell plates at a cell concentration that would yield no more than 1×10^6 cells/mL on the day of harvest, assuming that the cells double approximately every 36 hr. For example, a cell suspension of 2×10^3 cells/mL was used to seed a series of multiwell plates; 12-well plates were inoculated with 5 mL/well for harvest in 8 days; 24-well plates were inoculated with 1.0 mL/well for harvest in 10–11 days, and so forth. The cells were given 1 day to attach and equilibrate prior to addition of ddC. Quadruplicate samples were prepared for each day that a measurement was taken and for each dosage of ddC. All samples were treated in parallel until harvest. Lactate analysis and cell counts were performed on each sample, and the results were averaged. Every 48 hr or less, the culture medium was removed and replaced with fresh medium containing the appropriate drug concentration. Measurements were timed such that when one set of samples was harvested, the medium was replaced in the remaining samples.

In induced GS-*ras*-2 cells, lactate production was analyzed in the same manner except that wells were precoated with poly-L-lysine [7] to maximize neurite outgrowth and that dexamethasone (0.075 μM) was added on day 1 after seeding the wells and ddC on day 2.

Measurement of O_2 Uptake

A single 100-mm culture dish was seeded with 0.55 to 1.0×10^6 cells in 20 mL of medium for each time point desired. The cells were given 1 day to attach and equilibrate. Every other day the medium was aspirated and replenished with 10 mL of fresh medium containing the appropriate concentration of drug. All of the samples in a single experiment were harvested on the same day so that 10 μM ddC was added to selected dishes 6, 12, 24, and up to 132 hr prior to harvest. At harvest, the medium was aspirated after reserving a fraction for lactate analysis, and the monolayer was washed once with 5 mL of phosphate-buffered saline (138 mM NaCl, 2.67 mM KCl, 10 mM Na_2HPO_4, 1.71 mM KH_2PO_4, pH 7.4). The cells were then washed off the plate using 3.7 mL of Krebs–Ringer solution [11] warmed to 37° and triturated by repeated pipetting to reduce clumping. Then 3 mL of cell suspension was introduced into a polarographic apparatus (Yellow Springs Instrument Co., Yellow Springs, OH) at 37°, and O_2 consumption was measured with a Clarke-type electrode. A 200-μL fraction of the residual suspension was removed and stored at 4° for determination of the cell count. Cells were counted using a Coulter counter as described earlier [7].

Measurement of Respiratory Control

Rat liver mitochondria were isolated from Sprague-Dawley rats. Analysis of respiratory control was performed according to the method of Estabrook [12] except that the oxidizable substrate used was α-ketoglutarate (added in the form of potassium glutamate), triethanolamine buffer was replaced by Tris, and O_2 measurements were made polarographically as described earlier.

Measurement of Lactate per Cell

For lactate determination, 400 μL of medium was transferred from the cell culture to an Eppendorf tube containing 800 μL of cold 10% trichloroacetic acid. The mixture was incubated on ice for 10 min, spun in a microfuge for 10 min, and then 1 mL of the supernatant was stored at −80° pending assay. Measurement of lactate concentration was performed using a pyruvate-lactate diagnostic kit (826-A) from the Sigma Chemical Co. (St. Louis, MO) and following the manufacturer's directions. Spectrophotometric determination of NADH at 340 nm was used to follow the lactate dehydrogenase-catalyzed reduction of NAD.

RESULTS

Effect of ddC on Lactate Production in the Undifferentiated Proliferating PC12 Cell

The classical cellular response to interference with ATP production, whether by an uncoupling of oxidative phosphorylation or by inhibition of terminal electron transport, is to compensate for decreased ATP level by increasing ATP production via glycolysis. This leads to an increase in the rate of production of lactic acid. Such an increase was found when the Friend murine erythroleukemic cell was treated with AZT; the rate of lactic acid production started to rise within 3 hr [5]. The results of exposure of uninduced

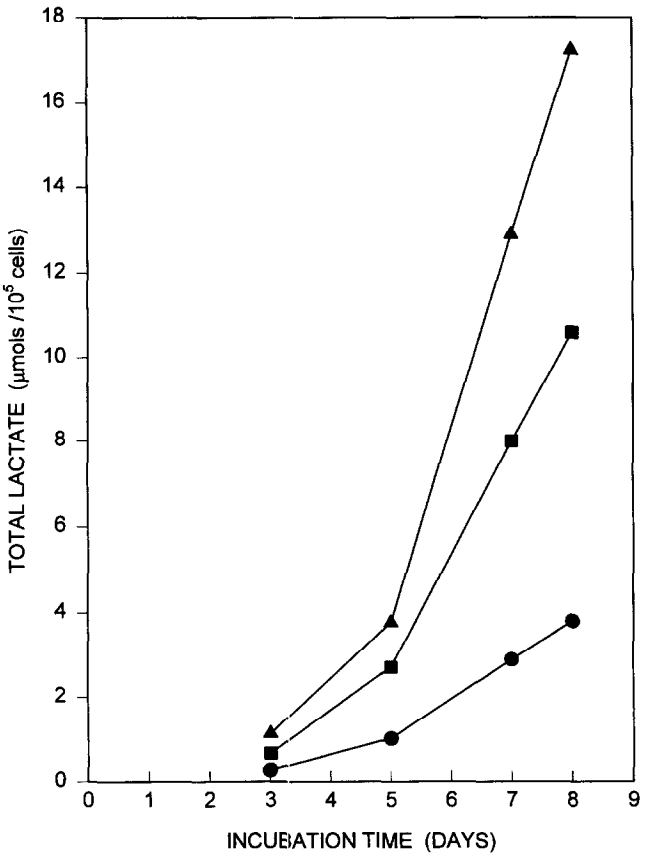

FIG. 1. Effect of addition of ddC to the growth medium on lactate production by undifferentiated PC12 cells. The PC12 cells (transformant GS-*ras*-1) were not exposed to dexamethasone. Other experimental conditions are given in Materials and Methods. This experiment was performed three times, and the results are in accord with those of the other experiments. The values of the blank (no ddC) samples were subtracted prior to plotting the curves; for 3, 5, 7, and 8 days, they were 0.1, 0.7, 2.4, and 3.4 μmol/10^5 cells, respectively. ddC was added at day 1 and its concentrations were: (●—●) 10 μM; (■—■) 25 μM; and (▲—▲) 50 μM.

PC12 (GS-*ras*-1) cells (i.e. cells not exposed to dexamethasone) to various concentrations of ddC are shown in Fig. 1. Since a small amount of lactic acid is produced even in the absence of ddC, these blank values were subtracted from the experimental values; the blank values are given in the legend.

The results show a concentration-dependent response of lactate formation to ddC concentrations of 10, 25, and 50 μM ddC, concentrations chosen on the basis of yielding good inhibition of mtDNA replication. In addition, it is evident that a considerable period of time elapsed between the addition of ddC (at day 1) and the rise in lactate concentration. In the case of 10 μM ddC, a concentration that takes about 4–5 days to produce inhibition of cell proliferation, it can be seen that it took about 2 days of exposure to ddC before any sign appeared of lactate increase, and in some experiments the delay was often a day longer than this. (See Fig. 3 in which earlier points are shown.) Even at the higher concentrations of 25 and 50 μM ddC, concentrations far exceeding pharmacological levels, there was little effect at day 2; the initiation of the steep rise occurring 4 days after the ddC exposure.

Thus, these results offer no support for an immediate, early, or direct action of ddC on oxidative phosphorylation. Rather, they are consistent with an indirect effect, namely, a prior inhibition of mtDNA replication by ddC, which we have shown to occur [8]. Thus, after several cell generations, a point is reached at which the number of mtDNA-encoded protein components of the oxidative phosphorylation system becomes limiting, ATP in turn becomes limiting, and lactic acid production begins to increase.

Effect of ddC on Lactate Production in the Differentiated (Non-proliferating) Cell

Upon addition of dexamethasone to the GS-*ras*-2 cell, cell proliferation ceases and neurite outgrowth commences. The results of exposure of such differentiated cells to ddC on lactate formation are shown in Fig. 2. A concentration-related response to a range of ddC concentrations between 2.5 and 50 μM was observed. Our previous results on such cells, along with pilot experiments, indicated that commencement of inhibition of neurite outgrowth occurred after a long delay, about 10 days after initiation of exposure to 12.5 μM ddC [8]. (Note that ddC treatment started at day 2.) It thus seemed reasonable to examine lactate production starting at a later incubation time than that shown in Fig. 1. It can be seen that an appreciable delay occurred in lactate production (Fig. 2). At 5 days, a rise in lactate concentration had not yet started at ddC levels of 2.5 and 5 μM and was just beginning at 10 μM. Lactate concentrations were still very low at this time, even at 25 and 50 μM ddC. It is of interest that lactate production increased drastically at 10 days after ddC exposure, at about the same time that neurite output was inhibited. Thus, the delay in the initiation of lactate formation again speaks against a direct or early effect of ddC on oxidative phosphorylation, in contrast to the action of AZT. Rather, such a delay points to an indirect effect stemming from an inhibition of replication, and in favor of an indirect effect mediated by the decreased rate of mtDNA replication.

Does the Rise in Lactate Production Result from an Uncoupling of Oxidative Phosphorylation or from an Inhibition of the Rate of the Coupled Process?

If defective mtDNA replication results, in part, in new mitochondria possessing uncoupled oxidative phosphorylation systems, such uncoupling should produce an increase in O_2 consumption, a classical sign of uncoupling. On the other hand, if oxidative phosphorylation is inhibited at some point not involving uncoupling, for example by an inhibition of the rate of terminal electron transport, a decrease rather than an increase in O_2 consumption should be observed.

Uninduced cells were exposed to 10 μM ddC, and O_2

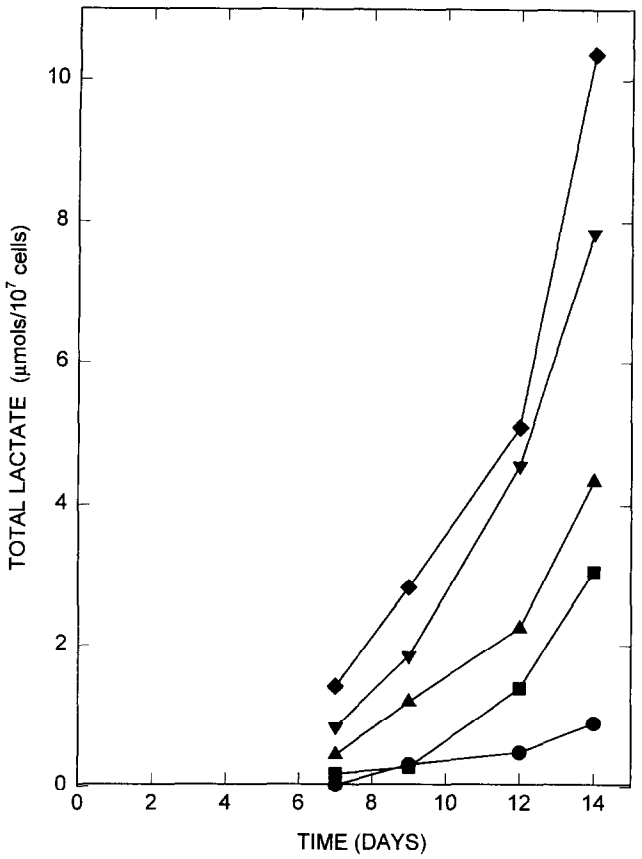

FIG. 2. Effect of addition of ddC to the growth medium on lactate production by differentiated PC12 cells. The PC12 cells (transformant GS-*ras*-2) were induced to differentiate with dexamethasone added at day 1. This experiment was performed three times, and the results are in accord with those of the other experiments. The values of the blank (no ddC) samples were subtracted prior to plotting the curves; for 7, 9, 12, and 14 days, they were: 0.43, 0.61, 1.2, and 2.0 μmol/10^7 cells, respectively. ddC was added at day 2, and its concentrations were: (●—●) 2.5 μM; (■—■) 5 μM; (▲—▲) 10 μM; (▼—▼) 25 μM; and (◆—◆) 50 μM.

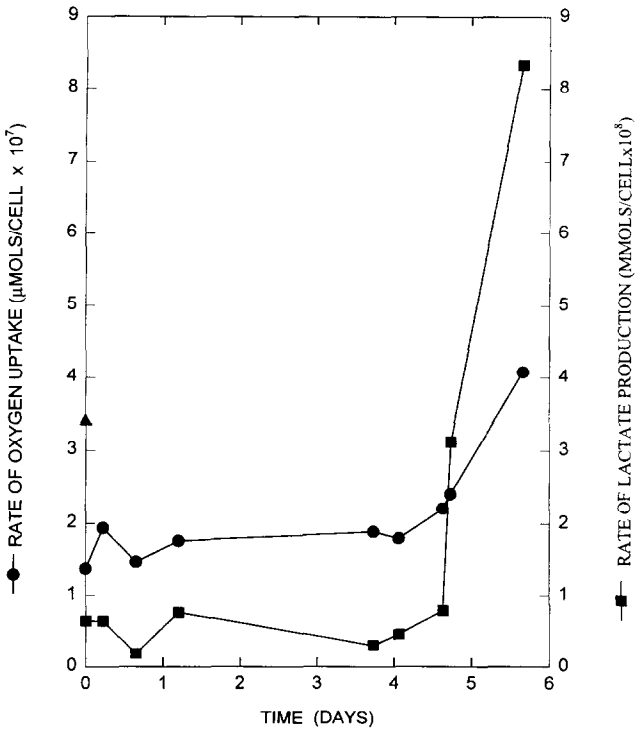

FIG. 3. Effect of ddC on O_2 uptake by PC12 cells and correspondence between O_2 uptake and lactate production. The PC12 cells (transformant GS-*ras*-1) were not exposed to dexamethasone. This experiment was performed twice, and the results are in accord with those of the other experiment. ddC was added at day 1, and its concentration was 10 μM. Key: (■—■) lactate; (●—●) O_2; and (▲) O_2 uptake 15 min after the addition of uncoupler (*p*-trifluorocarbonylcyanide methoxyphenylhydrozone, 5 μM).

consumption and lactate formation were measured. The plot of rates of lactate formation and O_2 consumption (Fig. 3) shows that again there was a rise in the rate of lactate formation and a concomitant rise in the rate of O_2 uptake, both delayed for about 3 days (ddC was added at day 1) and both rising steeply thereafter. As a control, we measured the effect of treating the cells with 5 μM *p*-trifluorocarbonylcyanide methoxyphenylhydrazone, a known uncoupler, for 15 min, sufficient time for complete uncoupling to occur [13]. The uncoupler caused an increase in O_2 uptake similar to that brought about by ddC by day 5.7. These are the results that would be expected if oxidative phosphorylation were uncoupled, and the fact that the increases occur concomitantly strengthens the argument that uncoupling has indeed occurred. However, the delayed effect of ddC on the uncoupling is consistent with the view that ddC does not directly execute the uncoupling but instead acts through its impairment of mtDNA replication and, therefore, the synthesis of proteins required for oxidative phosphorylation.

Direct Evidence That ddC Is Not an Uncoupler of Oxidative Phosphorylation; Effect of ddC on a Classic Respiratory Control System

When a tightly coupled oxidative phosphorylation system becomes uncoupled, it loses its capacity for respiratory control, that is, O_2 consumption becomes freed from control by ADP. Thus, whether or not a substance is an uncoupler can be ascertained by determining whether its presence in such a system impairs the ability of added ADP to stimulate O_2 uptake. Moreover, if the substance is indeed an uncoupler, its addition itself should stimulate oxygen uptake. The "standard" system for such measurements is isolated intact rat liver mitochondria incubated in a medium containing an oxidizable substrate; α-ketoglutarate added as glutamate was used in our experiments.

Evidence that our *in vitro* respiratory control system was operating properly came from two experiments. We first tested the effect of the addition (at 6 min) of ADP (Fig. 4). This resulted in a 3-fold increase in the rate of O_2 uptake, an acceptable respiratory control index. Second, to an otherwise identical sample run in parallel (not shown), the

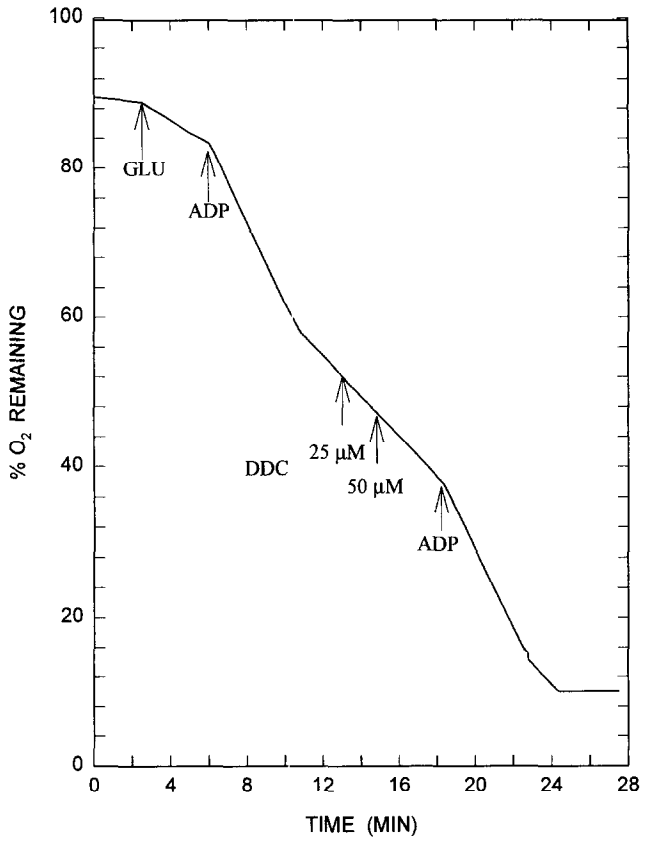

FIG. 4. Effect of ddC on respiratory control in isolated rat liver mitochondria (the "standard" respiratory control system). This experiment was run twice, and the results are in accord with those of the other experiment. Rat liver mitochondria were suspended in Krebs–Ringer phosphate incubation solution, and their O_2 uptake was measured polarigraphically. The reaction mixture was equilibrated with air, and the equilibration point was set arbitrarily at 100% saturation, a value equivalent to 1440 μatoms of O_2. The concentrations used of potassium glutamate and ADP were 10 and 0.3 mM, respectively. Other experimental conditions are given in Materials and Methods. The graph shows the recorder curve. Arrows indicate the points of addition of the indicated substances. To an otherwise identical sample run in parallel (not shown), the uncoupler 2,4-dinitrophenol (final concentration 20 μM) was added in place of ddC (see Results).

classical uncoupler 2,4-dinitrophenol (final concentration 20 μM) was added in place of ddC. The addition of the uncoupler caused a sharp 2.5-fold stimulation of O_2 uptake (similar to that shown in Fig. 5 of Ref. 5). To test the effect of ddC (Fig. 4), we waited until the added ADP was exhausted before adding ddC. It can be seen that neither the addition of 25 μM ddC at about 13 min, nor that of 50 μM ddC at about 15 min led to increased O_2 uptake. Moreover, this total of 75 μM ddC did not affect respiratory control by ADP, added at about 18.5 min; the extent of the stimulation of the rate of O_2 uptake by ADP in the presence of ddC was the same as that in its absence (6 min). The results thus show clearly that ddC in itself was not an uncoupler of oxidative phosphorylation.

DISCUSSION

To examine the biochemical mechanisms underlying the induction of peripheral neuropathy by ddC/ddI/d4T, we have chosen, in this and previous studies [7, 8], the PC12 cell. This cell line is derived from a rat pheochromocytoma, an adrenal medullary tumor. Since adrenal medulla cells (including PC12 cells) share their embryological origin with neuronal cells, they possess many of the properties of neuronal cells including neurite outgrowth upon NGF-induced differentiation. We used the more convenient GS-*ras*-1 and GS-*ras*-2 transformants of this cell, and these are described in Materials and Methods.

Impairment of Oxidative Phosphorylation

To link the ddC-induced inhibition of mtDNA replication [8] (with its consequent depletion of mtDNA) more closely to the induction of peripheral neuropathy, it needs to be demonstrated that such inhibition of replication of the mitochondrial genome leads, in a relevant cell, to a metabolic abnormality severe enough to disrupt normal cellular function. Our results show that such an effect did indeed occur, namely a serious impairment of oxidative phosphorylation both in uninduced cells and in cells induced to differentiate and produce neurites. Such a result is not unexpected, considering that the mitochondrial genome codes for 13 oxidative phosphorylation proteins. That severe mtDNA depletion results from exposure of proliferating cells to inhibitors known to suppress both mtDNA proliferation and DNA polymerase γ is supported by results on cell lines from different species with different inhibitors. First, it has been shown that inhibition of mtDNA replication by the non-ddN inhibitor ethidium bromide [14, 15] (using a human cell line) and by the ddN inhibitor AZT* [1, 3] (using the murine erythroleukemic cell) leads to a drastic loss of mtDNA. More relevant evidence comes from the demonstration [9] that, in the PC12 cell, even a ddC concentration as low as 0.5 μM substantially decreases the level of mtDNA; 5 μM drastically decreases it, while 50 μM depletes it virtually completely. A similar range of concentrations (2.5, 5, 10, 25, and 50 μM) were used in our studies.

Delayed Inhibition of Oxidative Phosphorylation

The results of the present study also show that, unlike AZT, ddC does not show an immediate or early effect on oxidative phosphorylation; AZT manifests its effect as early as 3 hr [5]. Rather, the results are consistent with the view that this process is impaired through the prior inhibition by ddC of mtDNA replication. In support of this view are the results showing that ddC itself is not an uncoupler of oxidative phosphorylation; the observed uncoupling was probably brought about by the inhibition of mtDNA

* Hobbs GA, Keilbaugh SA and Simpson MV, unpublished observations.

replication and the consequent failure of synthesis of some protein involved in oxidative phosphorylation. The possibility cannot be eliminated, however, that the difference we observed in the action of the two nucleotides results from the different cell types used, the Friend cell in the case of AZT and the PC12 cell in the case of ddC. This possibility is unlikely, however, inasmuch as the oxidative phosphorylation systems in all mammalian cell types are very similar.

A sizable decrease in the rate of oxidative phosphorylation is likely to derange metabolism sufficiently to cause many metabolic problems for the cell even in the face of supplementation of ATP via increased glycolysis. Moreover, the impairment of the mitochondrial genome is likely to have deleterious consequences in addition to the direct inhibition of ATP synthesis, since the proteins encoded by the genome are also components of the structure of the internal mitochondrial membrane. It thus seems reasonable that the impairment of this membrane to any significant degree may well have consequences on mitochondrial functions other than ATP synthesis.

For example, it has been mentioned earlier that growth of cells in ethidium bromide, an inhibitor of DNA polymerase γ [14], can lead to a complete loss of mtDNA [15]. Such cells are, of course, defective in oxidative phosphorylation. Their mitochondria, however, also become impaired in an additional function; a normally present nuclear DNA encoded enzyme responsible for a step in pyrimidine synthesis is missing [15]. Supplementation of the medium with uridine (plus pyruvate) permits cell growth to continue. Evidence that ddC-treated PC12 cells also suffer such an impairment comes from experiments that show the efficacy of uridine in alleviating the deleterious effect of ddC on their growth and viability [8].

Another example, one particularly pertinent to the role of ddC in inducing peripheral neuropathy, involves the part played by mitochondria in Ca^{2+} metabolism. Mitochondria have been shown to control Ca^{2+} levels in the cytoplasm (even in discrete areas of the cell) [16] by taking up and releasing Ca^{2+}. Ca^{2+} uptake in mitochondria is energized by the proton gradient across the mitochondrial membrane established by the mitochondrial terminal electron transport system [17, 18]. It has been shown recently [19] that treatment of dorsal root ganglion cells with ddC lowered mtDNA levels and decreased the mitochondrially mediated component of depolarization-induced Ca^{2+} transients, thus decreasing the direct buffering of Ca^{2+} by mitochondria, a situation bound to have serious consequences for the functioning of this neuronal cell. (Some of the studies cited in the next section are also examples of effects of ddC not likely to be mediated by inhibition of mtDNA replication.)

Finally, and again particularly germane to the problem of peripheral neuropathy, are findings from a number of laboratories that specific mutations in mitochondrial genes are linked to various neuropathies and neurodegenerative disorders [20–23].

Some Differences in the Action of ddC and AZT

The contrast in the abilities of AZT and ddC to exert an early (direct?) effect on oxidative phosphorylation is not the only way in which the effects of ddC and AZT differ. Indeed, the two ddNs function differently in many ways. To start with, in contrast to the similar manner in which both drugs interact with HIV-1 reverse transcriptase (incorporation into DNA resulting in DNA chain termination), they act quite differently from each other on the mitochondrial enzyme DNA polymerase γ. ddC inhibits the action of DNA polymerase γ primarily by chain termination, whereas AZT acts primarily as a competitive inhibitor rather than as a substrate [24, 25] (although some incorporation of AZT must occur inasmuch as an exonuclease has been found which has been shown to cleave its residue from the mtDNA of AZT-treated cells [26]). Such a difference could have metabolic consequences. In the latter case, the rate of DNA replication would decrease with possibly all genes surviving. In the former, some genes, particularly those farthest from the origins of replication, might not survive at all.

AZT and ddC show a number of other differences in behavior. One such effect is the conversion of AZT by the liver to 3′-amino-3′-deoxythymidine, itself highly toxic to cells [27, 28]. In addition, both aminothymidine and to a lesser extent AZT itself, in their monophosphorylated forms, have been shown to inhibit the 3′ to 5′ exonuclease (proofreading) activity of calf thymus DNA polymerase δ. This would be expected to result in a more rapid rate of mutation of nuclear DNA in humans if the human enzyme were similarly inhibited [29].

It has also been shown that in proliferating cells exposed to AZT, a high proportion of guanosine residues in mtDNA become converted to 5-hydroxyguanosine [30]. Such mutations could result in the synthesis of defective mitochondrial proteins, thereby disrupting mitochondrial structure and function. To our knowledge, the guanosine oxidation reaction has not been reported for non-azidylated ddNs.

AZT also has the ability to stimulate mitochondrial proliferation in the Friend cell [4] and in human muscle *in vivo* [31]. (The new mitochondria, however, possess less DNA per mitochondrion.) We have not observed this phenomenon when using ddC.

It has been reported that AZT inhibits the transcription of the globin gene in the K-562 leukemia cell [32] while the expression of three other erythroid inducible genes is not affected [33]. The possibility has not been eliminated, however, that this inhibition results from a mitochondrial inner membrane defect arising from AZT inhibition of mtDNA replication and that this, in turn, leads to a disturbance of that part of the heme synthesis pathway which is localized in mitochondria, thereby leading to an inhibition of heme synthesis. Since globin synthesis is positively regulated by heme, a decrease in heme concentration would decrease globin production and most likely lead to a decline in globin messenger synthesis.

Finally, the two nucleotides differ in their predilection for different cells. AZT attacks the Friend murine erythroleukemic cell but not the PC12 cell. Reciprocally, ddC (as well as ddI and d4T) attacks the PC12 cell but has little or no effect on the Friend cell [4, 7], and this accurately reflects the differences in human side-effect selectivity in response to these drugs.

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