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RAPID COMMUNICATION

THE PC12 CELL AS A MODEL FOR STUDIES OF THE MECHANISM OF INDUCTION OF PERIPHERAL NEUROPATHY BY ANTI-HIV-1 DIDEOXYNUCLEOSIDE ANALOGS

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The ability of DNA polymerase gamma to utilize ddNTPs[+] as substrates, incorporating these chain terminators into DNA (see citations in [1]), has led us to propose that AZT-induced bone marrow suppression results from inhibition of mtDNA replication [1]. This could alter metabolism since mtDNA encodes proteins involved in ATP synthesis and mitochondrial ultrastructure. We have shown that AZT and some other ddNs used in AIDS therapy inhibit DNA replication by purified DNA polymerase gamma and by isolated mitochondria [1,2]. Moreover, proliferation of a hemopoietic cell, the Friend erythroleukemic cell, is strongly inhibited by AZT [2], and mitochondria from these cells show impairment of DNA replication[++] [2]. Additional evidence comes from studies on the effect of ddC on the Molt-4F cell [3], and on the involvement of mitochondria in AZT-induced myopathy [4].

We propose that inhibition of mtDNA replication is also the primary step in ddC-induced [5], ddI-induced [6] and d4T-induced [7] peripheral neuropathy. We have found that a c-Ha-ras transformant of the PC12 cell, GS-ras-1[+++], is a promising neuronal cell model for such studies. The PC12 cell line [8] is derived from a pheochromocytoma, an adrenal medullary tumor; cells of the adrenal medulla share their embryological origin with neuronal cells. When the PC12 cell is induced by NGF (or by dexamethasone in GS-ras-1) to differentiate, neurite outgrowth and increased synthesis of acetylcholine and other neuronal markers occur. We describe the effects of some ddNs on both the uninduced and the induced cell.

MATERIALS AND METHODS

GS-ras-1 cells were maintained in tissue culture dishes in D-MEM (GIBCO Cat. No. 320-1965PJ), horse serum, and fetal bovine serum (90:10:5) at 37°, 10% CO_2 and 98% humidity. Cultures were passed every five days (maximum cell density, 2.5 x 10^5-1.0 x 10^6 cells/mL). For the experiments, one 20-mL culture was treated with 5 mL of 0.05% trypsin containing 5 mM $MgCl_2$ for 2.5 min at 37°, diluted with 15 mL of PBS [9], centrifuged at 200 g for 4 min, resuspended in fresh medium, and triturated prior to passage at the dilutions noted below. Extent of trituration, forcing the cell suspension through a pipet tip held nearly flat against a culture dish, varied from 5 times for passing to 25 times for determination of neurites/cell.

Cultures used for growth studies were passaged at about 1.5 x 10^3 cells/mL into 24-well plates and were incubated for 24 hr prior to drug addition to permit cell attachment. The medium was replenished on day 4 or 5 after passage and every other day thereafter: 80% of the spent medium was removed and replaced with an equal volume of fresh medium containing a 25% greater concentration of drug on the assumption that all the previous dose had been metabolized. Were no drug lost, only a moderate increase would occur in drug concentration after at the most 3 medium changes. The fresh medium was slowly discharged directly toward the well wall, allowing it to flow down to and gently over the cells; few if any cells became detached. Cells were considered viable if they remained attached to the plate during incubation. At harvest the cells were resuspended in their medium, transferred to an Eppendorf tube, centrifuged at 2500 rpm for 4 min in a microfuge, washed once with 1 mL of

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[+]Abbreviations: HIV, human immunodeficiency virus; ddNTP, dideoxynucleoside triphosphate; AZT, 3'-azido-3'-deoxythymidine; mtDNA, mitochondrial DNA; ddN, dideoxynucleoside; AIDS, acquired immunodeficiency syndrome; ddC, dideoxycytidine; ddI, dideoxyinosine; d4T, 3'-deoxythymidine-2'-ene; NGF, nerve growth factor; D-MEM, Dulbecco's Modified Eagle Medium; PBS, phosphate buffered saline; and dCyd, deoxycytidine.
[++]Hobbs G, Keilbaugh SA, Prusoff WH and Simpson MV, manuscript in preparation.
[+++]Kremer NE, D'Arcangelo G, DeMarco M, Brugge JS and Halegoua S, Signal transduction by nerve growth factor and fibroblast growth factor requires a cascade of src and ras actions, submitted.

PBS, resuspended, and treated with 150 μL of 0.05% trypsin containing 5 mM $MgCl_2$ at 37° for 15 min. Following thorough trituration, cell counts were determined on a Coulter Counter.

The 2.5-mL cultures used to determine neurites/cell were passaged at about 200 cells/mL into 35-mm dishes precoated overnight with poly-L-lysine [10]. After incubation for 15 hr, 1.3 μL of 1 mM dexamethasone in dimethyl sulfoxide (DMSO) was added, followed 15 hr later by drug addition. Every 4 days the culture medium was replaced with 2.5 mL of fresh medium containing dexamethasone and drug. Cell and neurite counts were done in a statistically significant manner using phase contrast microscopy at 200x. We defined a neurite as a process of 2 or more cell bodies in length possessing an attachment foot.

RESULTS AND DISCUSSION

The first phase of our experiments involving undifferentiated cells revealed a sharp difference between ddC and AZT (Figs. 1 and 2). ddC inhibited cell proliferation in a concentration dependent manner (Fig. 1). At 50 μM ddC, complete inhibition of cell growth was always observed by day 10 (experiments not shown) but, for unknown reasons, the onset of this effect varied from experiment to experiment and could be as early as 4 or 7 days (Figs. 3 and 4). Appreciable inhibition was always evident even at 12.5 μM ddC (Figs. 1, 3 and 4). The small inhibition seen at 5 μM ddC (Fig. 1) was observed in some, but not all, replicate experiments. The effect of low ddC levels at longer incubation times is being studied. Note that the inhibitory effect of 50 μM ddC was reversed completely by an equal concentration of dCyd, which speaks against ddC acting as a general cytotoxic agent.

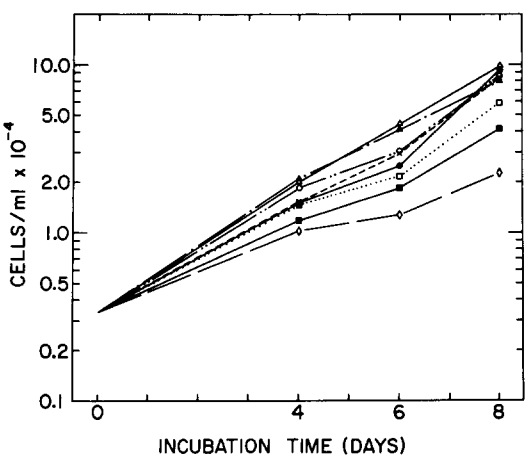

Fig. 1. Effect of ddC on PC12 (GS-ras-1) cell growth. Cells were grown in 0 (●—●), 1 (○···○), 5 (×-×), 12.5 (□···□), 25 (■—■), 50 (◇--◇) μM ddC; 50 μM dCyd (△—△) and 50 μM dCyd + 50 μM ddC (▲···▲). Each point is the average of triplicate samples.

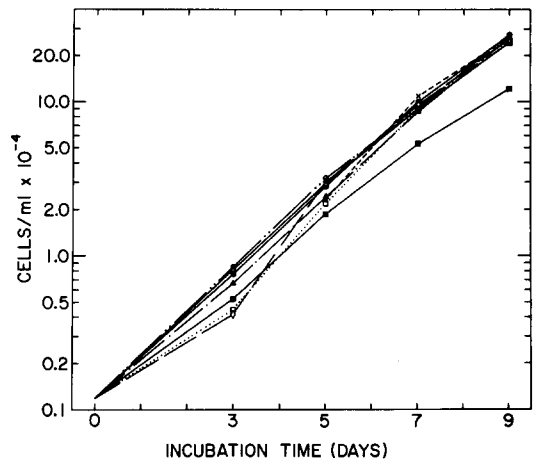

Fig. 2. Effect of AZT on PC12 (GS-ras-1) cell growth. Cells were grown in 0 (●—●), 1 (◇--◇), 5 (×-×), 12.5 (□···□), 25 (▲···▲), 50 (△—△) μM AZT; 5 (○···○) and 25 (■···■) μM ddC. Each point is the average of triplicate samples.

AZT, in contrast to ddC, gave no hint whatsoever of cell growth inhibition, even at 50 μM (Figs. 2, 5, and many other replicate experiments). d4T and ddI exerted, at best, only small effects. In Figs. 3 and 5, the percent inhibition by 50 μM d4T was 29% (8 days) and 19% (9 days), respectively, and, in a third experiment (not shown), 27% (8 days). The degrees of inhibition for lower d4T concentrations await experiments with longer incubation periods. Inhibition by ddI is even more difficult to assess quantitatively, since the observed data (Figs. 4 and 5), insufficient to demonstrate reproducibility, suggest a very low inhibitory activity and only toward the end of the incubation period.

Figure 5 not only compares the effect of 50 μM drug concentrations in a single experiment, but also shows the results of an attempt to alleviate the effect of ddC with acetylcarnitine. If ddC causes ATP depletion due to partly impaired mitochondria, then supplementation of acetyl CoA pools might stimulate oxidative phosphorylation [11] in as yet unimpaired mitochondria. However, 100 μM acetylcarnitine had no effect whatsoever in reversing the inhibition by 50 μM ddC.

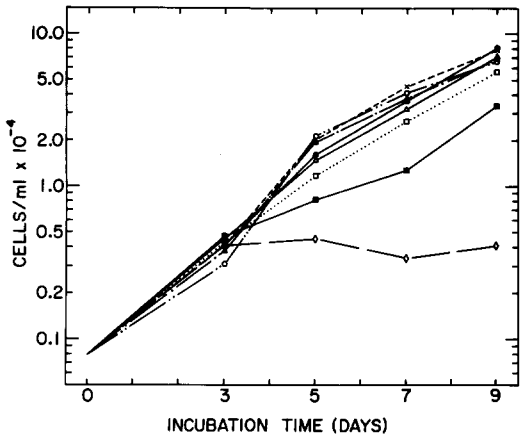

Fig. 3. Effect of d4T on PC12 (GS-ras-1) cell growth. Cells were grown in 0 (●—●), 1 (○···○), 5 (×-×), 12.5 (▲···▲), 25 (△—△), 50 (□···□) μM d4T; 12.5 (■—■) and 50 (◇--◇) μM ddC. Each point is the average of triplicate samples.

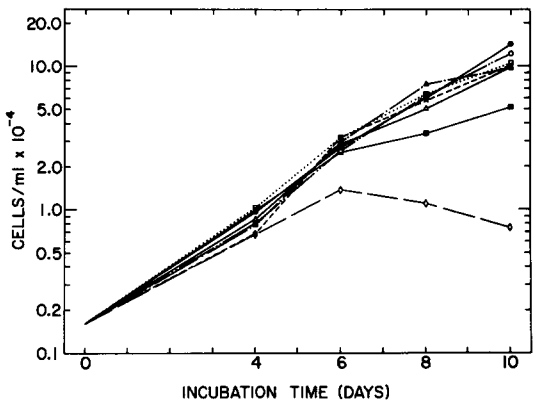

Fig. 4. Effect of ddI on PC12 (GS-ras-1) cell growth. Cells were grown in 0 (●—●), 1 (○···○), 5 (▲···▲), 12.5 (□···□), 25 (△—△), 50 (×-×) μM ddI; 12.5 (■—■) and 50 (◇--◇) μM ddC. Each point is the average of triplicate samples.

Since the neuronal properties of the undifferentiated cells are very limited, we hoped that the inhibitory effects noted above would be amplified in differentiating cells. On differentiation, cells stop replicating and begin outgrowth of neurites which is a distinct neuronal marker. Repeatedly, assays of neurites/cell (non-viable cells detach and are not scored) showed that the effect of ddC was dramatic (Fig. 6). After a marked stimulation by ddC, neurite outgrowth suddenly ceased at day 9, remained constant until day 15, and then dropped precipitously. Moreover, two noteworthy observations are not evident from these data. First, the structure of the neurites produced during the stimulatory phase was abnormal, and is currently under further investigation. Second, appreciable cell death began to occur at day 12 and accelerated markedly at day 15; at day 21 (not shown on graph), there were barely enough cells to count. In contrast, AZT exerted little or no

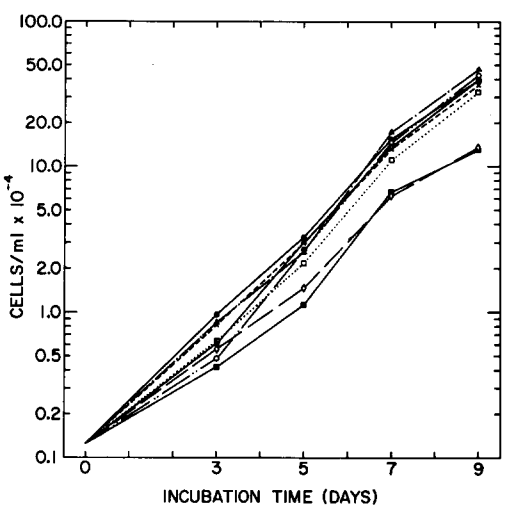

Fig. 5. Comparison in a single experiment of the effects of the four drugs on PC12 (GS-ras-1) cell growth. Cells were grown in 0 (●—●), 5 (○···○), 50 (◇--◇) μM ddC; 50 μM AZT (△—△); 50 μM ddI (×-×); 50 μM d4T (□···□); 100 μM acetyl-carnitine (▲···▲); and 50 μM ddC + 100 μM acetyl-carnitine (■—■). Each point is the average of triplicate samples.

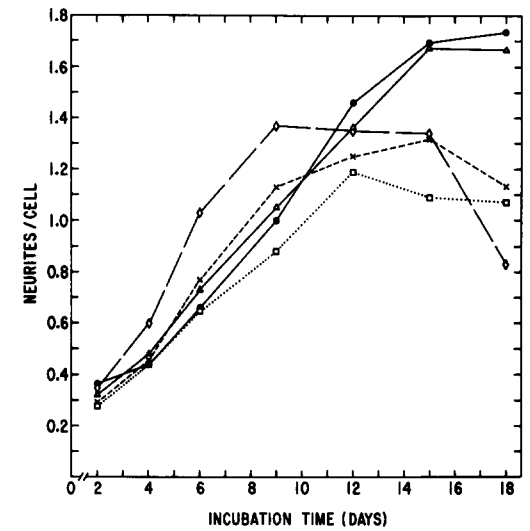

Fig. 6. Effects of ddC, AZT, ddI and d4T on differentiating PC12 (GS-ras-1) cells. Cells were induced with dexamethasone and were grown in 50 μM concentrations of the following drugs: control (●—●), ddC (◇--◇), AZT (△—△), ddI (×-×) and d4T (□···□). Each point is the average of triplicate samples.

stimulatory effect, neurites appeared normal, and no inhibition was evident. Both control and AZT curves began to plateau at about day 15. ddI exerted a small stimulatory effect at most during the first 9 hr, abnormal neurites were not observed, neurite production began to slow after day 9, and an inhibition in neurites/cell was apparent by day 12. While cell death began at day 15, the rate was far lower than in the ddC sample. We did not observe any stimulation with d4T and both the slowing of neurite outgrowth (day 6) and the decrease in neurites/cell began earlier than with ddI. Little difference between ddI and d4T was observed, however, in the neurites/cell endpoint values (day 18). As yet, the only distinction between the strengths of ddI and d4T is the preliminary, but consistent, observation that the d4T-treated differentiated cells are darker and glossier, making it much more difficult to visualize their nuclei. In summary, the results on the induced PC12 (GS-ras-1) cells make clear that ddC elicited by far the strongest response, ddI and d4T exerted fairly strong effects, and AZT had virtually no effect. In contrast, only ddC had a significant inhibitory effect on the undifferentiated PC12 (GS-ras-1) cells.

Of the dozen or so anti-HIV-1 ddN analogs tested on the Friend erythroleukemic cell, AZT is among those which exert the strongest effect [2], with ddC, d4T and ddI exerting a much weaker inhibition. This is in accord with clinical experience on the propensity of AZT to suppress bone marrow. On the other hand, clinical experience suggests that some of these drugs induce peripheral neuropathy in the order ddC>>>d4T≥ddI>>AZT, with AZT producing virtually no effect. It is evident from our results, therefore, that the PC12 cell, or at least its ras transformant, may be a useful system for the study of the role of ddN-induced peripheral neuropathy as well as for the development of a drug-screening system. It is not yet clear whether the decrease in neurites/cell in the ddC sample toward the latter stages of the incubation period, 11 days earlier than in the control, occurred via neurite disintegration, preferential death of cells bearing higher numbers of neurites, neurite "shrinkage", or other factors. It is also important to determine which stage of the incubation period most closely reflects the in vivo induction of peripheral neuropathy.

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