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Insensitivity of Cultured Rat Cortical Neurons to Mitochondrial DNA Synthesis Inhibitors

EVIDENCE FOR A SLOW TURNOVER OF MITOCHONDRIAL DNA

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ABSTRACT. Mitochondrial dysfunction is a major contributor to aging and neurodegeneration. Defects in mitochondrial DNA (mtDNA) have been identified in several neuromuscular diseases. Even though there is a high rate of phenotypic expression of mtDNA mutations in the central nervous system and replication of DNA introduces errors, little is known about the replicative activity of mtDNA in the brain. In this study, we investigated the sensitivity of cultured rat cortical neurons to mtDNA synthesis inhibitors as a means to assess the turnover rate of mtDNA. Four-day treatment with dideoxycytidine (ddC) (0.2 μM) or ethidium bromide (EtB) (0.25 μg/mL) reduced the mtDNA content approximately 80% in the human lymphoblastoid cell line, CEM. Concentrations of ddC ranging from 0.2 to 10 μM did not reduce mtDNA content in primary cultures of rat cortical neurons. Similarly, treatment with EtB (0.1, 0.25, and 0.5 μg/mL) did not affect significantly neuronal mtDNA. EtB (0.25 μg/mL) was effective in reducing mtDNA content in the undifferentiated embryonic carcinoma cell line, P 19. However, once P 19 cells were differentiated into a neuronal phenotype, they became insensitive to inhibition of mtDNA synthesis by EtB. Thus, cultured rat cortical neurons were less sensitive to mtDNA synthesis inhibitors than cell lines, suggesting that the turnover of mtDNA in central neurons is very slow. This may protect central neurons from accumulating mutations during the replication of mtDNA. BIOCHEM PHARMACOL **54**;1:181–187, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. mitochondrion; DNA replication; DNA turnover; cortical neurons; dideoxycytidine; ethidium bromide

Mitochondrial dysfunction is a major contributor to aging and neurodegeneration [1]. Impairment of cellular energy metabolism increases the susceptibility of neurons to glutamate, and thus represents an intrinsic determinant of the expression of glutamate neurotoxicity in a broad range of neurodegenerative disorders [2]. Because the mitochondrion contains its own DNA, heritable errors leading to mitochondrial dysfunction can arise from the mitochondrial genome as well as the nuclear genome. Defects in mtDNA† have been identified in several neuromuscular diseases, including Leber's hereditary optic neuropathy (LHON), ocular myopathies including chronic progressive external opthalmoplegia (CPEO), Kearns–Sayre syndrome, and myoclonus epilepsy with ragged-red fibers and stroke-like episodes [3].

mtDNA is a closed circular, double-stranded molecule approximately 16 kb in length. The human mitochondrial genome has been sequenced in its entirety and consists of genes that code for 13 subunits of the respiratory chain complexes, 22 transfer RNAs, and 2 ribosomal RNAs [4]. Each mitochondrion contains 2–10 copies of mtDNA. Whereas nuclear DNA synthesis takes place at a precise time in the cell cycle (the S phase), mtDNA synthesis appears to be continuous [5]. The enzyme responsible for mtDNA synthesis, DNA polymerase γ, is inhibited specifically by nucleoside analogs, such as ddC, which is an effective antiviral drug by virtue of its ability to inhibit reverse transcriptase [6]. The DNA intercalating molecule EtB also strongly inhibits mtDNA synthesis in a broad range of cell types ranging from yeast to human cell lines [7]. Using EtB, King and Attardi [8] have established human cell lines completely depleted of mtDNA (termed ρ^0 cells). These cells can be repopulated with exogenous mitochondria, thus providing an *in vitro* system for the analysis of mtDNA mutations and the study of nuclear–mitochondrial communication.

mtDNA exhibits a high mutation rate with an insertion error frequency for mtDNA polymerase γ of approximately 1 per 7000 bases, leading to 2–3 mismatched nucleotides per cycle of replication. This high mutation rate is related, at least in part, to its close apposition to the electron transport chain, the absence of a histone coat, and the

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† *Abbreviations:* AraC, cytosine arabinoside; ddC, dideoxycytidine; EtB, ethidium bromide; FBS, fetal bovine serum; and mtDNA, mitochondrial DNA.

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apparent lack of an adequate repair system [9]. Even though there is a high rate of expression of mtDNA mutation in the central nervous system and DNA replication introduces errors, little is known about the turnover of mtDNA in central neurons. Using Southern analysis, Chen *et al.* [6] have shown that treatment with ddC depletes mtDNA content in human cell lines and nerve growth factor-differentiated PC 12 cells. Using a similar protocol, Werth *et al.* [10] observed that ddC depletes mtDNA in cultured peripheral neurons, and Lewis *et al.* [11] found that mtDNA levels in skeletal muscle from rats treated with the nucleoside analog zidovudine are reduced. Inhibition of mtDNA replication with ddC should not affect mtDNA levels in cells that are not in the process of replicating mtDNA. Therefore, mtDNA must turn over in ddC-sensitive cells [12]. In this study, we investigated whether cultured rat cortical neurons were sensitive to inhibitors of mtDNA synthesis.

MATERIALS AND METHODS

Chemicals and Reagents

2′,3′-Dideoxycytidine, ethidium bromide, proteinase K, and RNAse A were purchased from the Sigma Chemical Co. (St. Louis, MO). Phenol was obtained from Ambion (Austin, TX). Chloroform was from Mallinckrodt Chemical (Paris, KY). *Bam*HI was from Promega (Madison, WI). [^{32}P]CTP and the random primer labeling kit were from Amersham (Arlington Heights, IL).

Cell Culture

Rat cortical neurons were grown in primary culture as previously described [13] with minor modifications. Fetuses were removed on embryonic day 17 from maternal rats anesthetized with CO_2 and killed by decapitation. Cortices were dissected and placed in Ca^{2+} and Mg^{2+}-free HEPES-buffered Hanks' salt solution (CMF-HHSS), pH 7.45. HHSS was composed of the following (in mM): HEPES, 20; NaCl, 137; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $MgCl_2$, 0.5; KCl, 5.0; KH_2PO_4, 0.4; Na_2HPO_4, 0.6; $NaHCO_3$, 3.0; and glucose, 5.6. Cells were dissociated by triturating through a 5-mL pipette and then a flame-narrowed Pasteur pipette. Cells were pelleted and resuspended in Dulbecco's Modified Eagle's Medium (DMEM) without glutamine, supplemented with 10% FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively). Dissociated cells were then plated at a density of 1.7×10^6 cells/flask onto 250-mL PRIMARIA flasks that had been coated with poly-*d*-lysine (0.1 mg/mL) and washed with H_2O. The neurons were grown in a humidified atmosphere of 10% CO_2 and 90% air (pH = 7.4) at 37°, and fed every 7 days by exchange of 30% of the medium with DMEM supplemented with 10% horse serum and penicillin (100 U/mL)/streptomycin (0.1 mg/mL). Cells were treated with 1 μM AraC at day 4 to reduce glial growth. AraC-treated cultures were composed of approximately 70% neuronal cells based on morphological criteria (a phase-bright rounded soma extending long, fine processes). Cortical neurons were treated with ddC or EtB on day 14 for 4 days. Fresh ddC and EtB were added every 2 days.

CEM, a human T lymphoblastoid cell line, was grown in RPMI 1640 medium supplemented with 10% FBS. To determine the effect of ddC or EtB, the cells (3×10^4 cells/mL) were treated with 0.2 μM ddC or 0.25 μg/mL EtB for 4 days with the addition of fresh drug every 2 days.

P 19 cells were cultured and induced to differentiate into a neuronal phenotype as previously described [14]. Briefly, cells were cultured in alpha-minimal essential medium (α-MEM) supplemented with 2.5% FBS and 7.5% calf serum in a 250-mL flask until confluent. The undifferentiated P 19 cells were then treated with 0.25 μg/mL EtB for 4 days with the addition of fresh EtB every 2 days. For differentiation, near-confluent cultures were trypsinized and dissociated. Then 1×10^6 cells were suspended in 10 mL α-MEM containing 5% FBS and 500 nM retinoic acid, and seeded onto a 10-cm bacteriologic culture dish. Cells were fed after 2 days by replacing the old medium with fresh medium containing retinoic acid. After day 4 of retinoic acid treatment, aggregates were trypsinized, dissociated, resuspended in α-MEM + 10% FBS, and plated onto a 250-mL flask containing confluent primary rat glial cultures at a density of approximately 10^7 cells/flask. On day 2 after plating, cells were switched to Eagle's MEM (Earl's salts) supplemented with 20 mM glucose, 5% FBS, and 5% horse serum (plating medium), and AraC (final concentration 20 μM) was added to inhibit the division of non-neuronal cells; the AraC was removed 2 days later by feeding cultures with plating medium. Cultures were subsequently maintained in this medium, and treated with 0.25 μg/mL EtB for 4 days.

mtDNA Measurement

The mtDNA probe Psp64 was provided by Dr. Susan P. LaDoux from the University of South Alabama. It encodes the entire mouse mtDNA sequence. The mtDNA content relative to total DNA content in cell samples was measured with the Southern blot technique as previously described [10]. Briefly, total cellular DNA was isolated by cell lysis and phenol chloroform extraction followed by digestion with ribonuclease A. DNA was digested overnight with *Bam*HI. Ten micrograms of *Bam*HI digested DNA isolated from control, ddC-, and EtB-treated cells was analyzed by gel electrophoresis on 0.7% agarose gel in TBE (0.089 M Tris-base–0.089 M boric acid–0.002 M EDTA) (pH 8.0) buffer containing 0.5 μg/mL EtB. Electrophoresis was carried out on a horizontal slab gel apparatus at 20 V overnight. After electrophoresis, the gel was exposed to UV light for 3 min and then soaked in 0.5 M NaOH/1.5 M NaCl to denature the DNA. After neutralization in 0.5 M Tris–HCl/0.5 M NaCl (pH 7.0) solution for 30 min, the gel was blotted onto nitrocellulose filters. The nitrocellulose filters were moistened with 6× SSPE (0.15 M NaCl/0.01 M

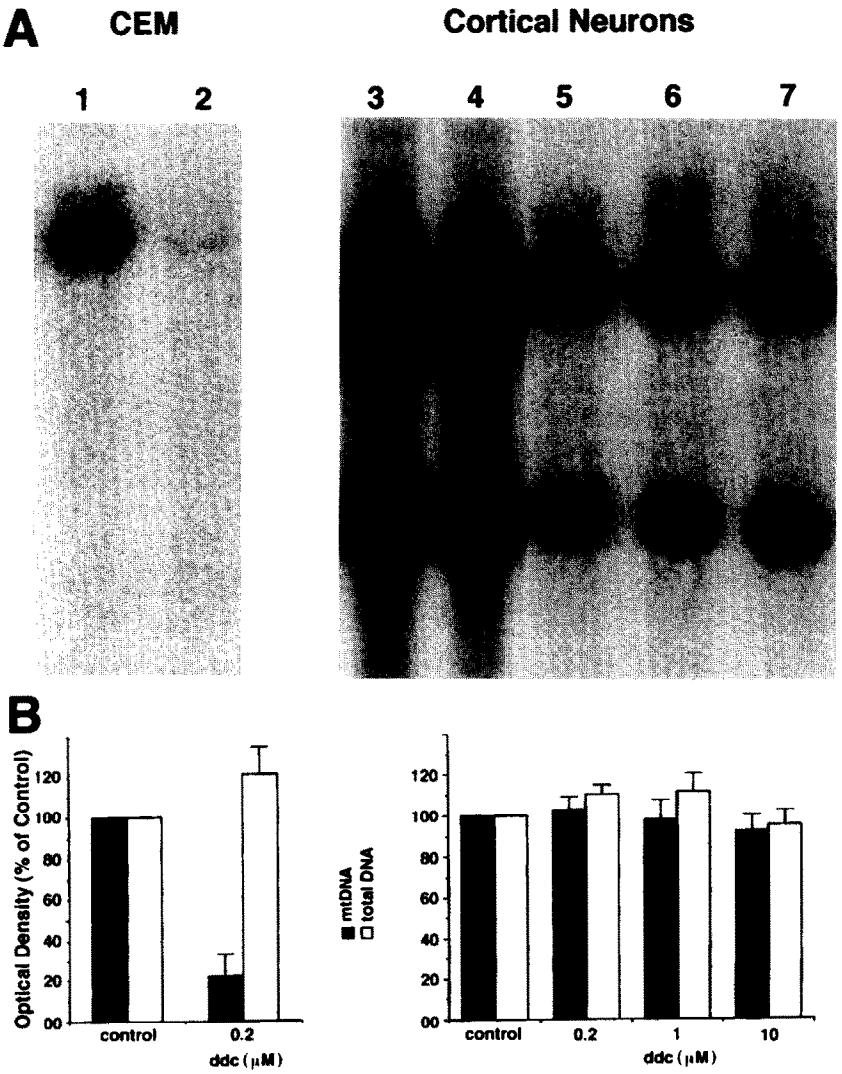

FIG. 1. Decrease of mtDNA content in CEM cells but not in cortical neurons by ddC. (A) Hybridization of mouse mtDNA probe to *Bam*HI fragments of total cellular DNA prepared from control and ddC-treated cells. Lanes: 1 and 2, CEM cells; 1, control; 2, 0.2 μM ddC; 3–7, cortical neurons; 3, control; 4, 0.2 μM ddC; 5, control; 6, 1 μM ddC; and 7, 10 μM ddC. (B) Quantitative analysis of total DNA and mtDNA in each sample of control and ddC-treated cells. Total DNA (open bars) and mtDNA (solid bars) were quantified by scanning the ethidium-stained gels and the autoradiograms, respectively, with a densitometer. Data represent means ± SEM from at least three experiments.

NaH_2PO_4/0.001 M EDTA, pH 7.4) containing 0.5% SDS and prehybridized for 2 hr at 62° in solution containing 50 μg/mL herring sperm DNA. Hybridization was carried out overnight at 62° in the same buffer with 25 ng of heat-denatured mtDNA probe-labeled with [^{32}P]dCTP. The filters were washed twice for 15 min each in 2× SSPE and 1× SSPE at room temperature, and in 0.5× SSPE at 65° with constant gentle agitation. Filters were exposed to Kodak X-Omat RP film and a Dupont Cronex Lightning-Plus intensifying screen at −70°. Total DNA and mtDNA in each sample were quantified by scanning the ethidium-stained gel and the autoradiograms with a GS-700 imaging densitometer with molecular analyst software from Bio-Rad Laboratories (Hercules, CA).

RESULTS

Depletion of mtDNA in CEM Cells but Not in Cortical Neurons by ddC

ddC has been shown previously to inhibit DNA polymerase γ, producing a corresponding decrease in mtDNA levels in the human lymphoblastoid cell line, CEM [6]. As shown in Fig. 1, we obtained similar results with these cells. Hybridization of the mouse mtDNA probe to *Bam*HI fragments from total DNA prepared from control and ddC-treated CEM cells showed a marked decrease in mtDNA content in drug-treated cells (Fig. 1A, lanes 1 and 2). Quantitative values for total DNA and mtDNA were obtained by densitometric scanning of the ethidium-stained gel (representing total DNA) and the corresponding autoradiogram (representing mtDNA only) (Fig. 1B). Thus, by loading equal aliquots of DNA in each lane, of which mtDNA is an insignificant amount in all lanes, we essentially normalized mtDNA to total DNA. Digestion with *Bam*HI linearized the human mtDNA, resulting in one band in the CEM lanes on the blot. Two bands were found in lanes loaded with rodent mtDNA. This results from the polymorphism of mtDNA in different species [6, 15]. Four-day treatment of CEM cells with 0.2 μM ddC reduced the mtDNA content in CEM cells by 78 ± 11% relative to control cells (Fig. 1A, lanes 1 and 2). Densitometric scanning of the

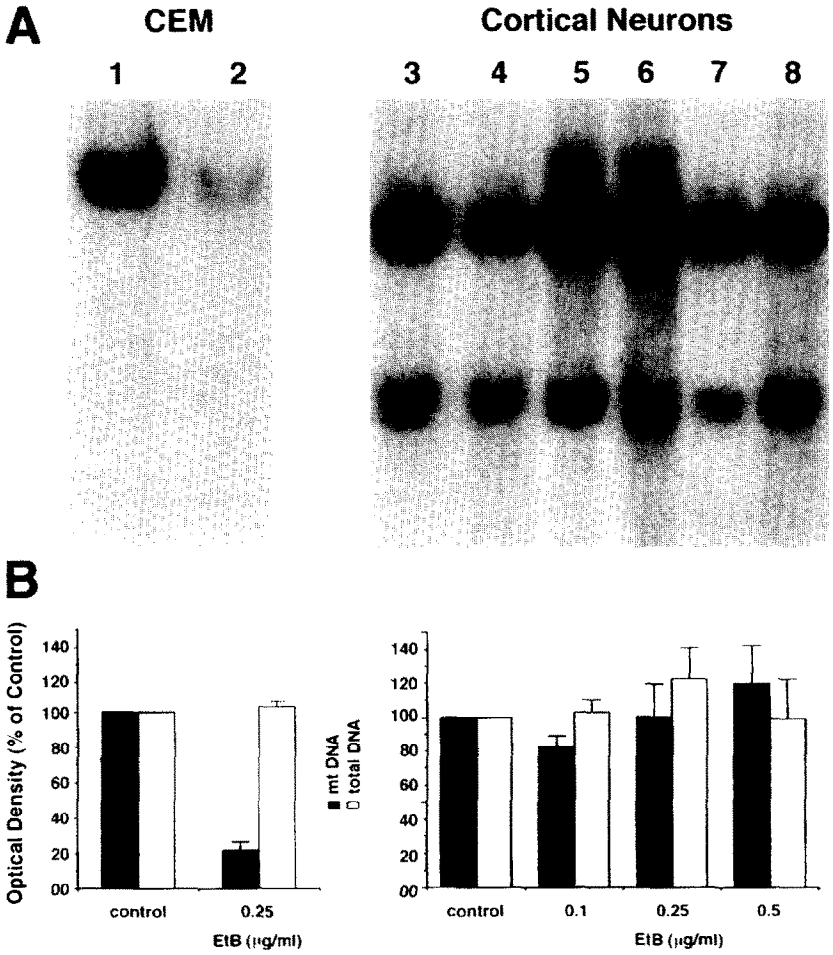

FIG. 2. Depletion of mtDNA content in CEM cells but not in cultured cortical neurons by EtB. (A) Hybridization of mouse mtDNA probe to *Bam*HI fragments of total cellular DNA prepared from control and EtB-treated cells. Lanes: 1 and 2, CEM cells; 1, control; 2, 0.25 μg/mL EtB; 3–8, cortical neurons; 3, control; 4, 0.1 μg/mL EtB; 5, control; 6, 0.25 μg/mL EtB; 7, control; and 8, 0.5 μg/mL EtB. (B) Quantitative analysis of total DNA and mtDNA in each sample of EtB-treated and control cells. Total DNA (open bars) and mtDNA (solid bars) were quantified by scanning the the ethidium-stained gels and the autoradiograms, respectively, with a densitometer. Data represent means ± SEM from at least three experiments.

ethidium-stained gel showed that the total DNA loaded in ddC-treated lanes was comparable to that in control lanes (Fig. 1B). Thus, consistent with the result of Chen *et al.* [6], 4-day treatment with 0.2 μM ddC depleted mtDNA in CEM cells. The decreased mtDNA content was not due to inhibition of cell growth because cell counts on day 4 of ddC treatment were similar in control and drug-treated cultures (control: 3.8×10^5 cells/mL; ddC-treated: 3.3×10^5 cells/mL). Thus, in CEM cells there is a continuous turnover of mtDNA. ddC, by inhibiting the synthesis of mtDNA, produced a net loss of mtDNA in these cultures. In contrast to the profound effects of ddC on mtDNA levels in CEM cells, the drug had no detectable effect on primary cultures of rat cortical neurons. ddC treatment identical to that applied to CEM cells (Fig. 1A, lanes 3 and 4) had no effect on mtDNA levels in cortical neurons. Increasing the ddC concentration to 1 and 10 μM still failed to affect mtDNA levels significantly in cortical neurons (Fig. 1, lanes 5–7). Since the total DNA amounts loaded for ddC-treated and control cortical samples were equivalent (Fig. 1B), it appears that cultured rat cortical neurons are insensitive to the mtDNA synthesis inhibitor ddC.

Depletion of mtDNA in CEM Cells but Not in Cultured Cortical Neurons by Ethidium Bromide

ddC exerts its inhibitory effect on mtDNA synthesis through its phosphorylated metabolite, ddCTP, which inhibits DNA polymerase γ. Transformation of ddC into ddCTP requires the sequential action of several cellular enzymes, including deoxycytidine kinase, cytosine nucleoside monophosphate kinase, and the nonspecific nucleoside diphosphate kinase [12]. The insensitivity of cortical neurons to ddC could result from a failure to convert ddC to ddCTP. For this reason, we tested the effect of another mtDNA synthesis inhibitor, EtB, on reducing mtDNA content in cortical neurons. EtB inhibits mtDNA synthesis by intercalating into DNA molecules. The preferential effect of EtB on mtDNA is probably due to the structural differences between circular mtDNA and linear nuclear DNA, and a high permeability of mitochondrial membranes to EtB [16]. EtB has been shown to inhibit mtDNA replication in yeast, mouse, hamster, avian, and human cell lines, indicating that it has effects on a wide range of cell types [7]. Four-day treatment of CEM cells with 0.25 μg/mL EtB reduced the mtDNA content by 78 ± 4% (Fig. 2A, lanes 1 and 2, and Fig. 2B). However, the same treatment

with various concentrations of EtB (0.1, 0.25, and 0.5 μg/mL) did not affect mtDNA content significantly in cultured cortical neurons (Fig. 2A, lanes 3–8, and Fig. 2B).

Reduction of mtDNA Content in Undifferentiated P 19 Cells but Not in P 19 Cells Differentiated into a Neuronal Phenotype by Ethidium Bromide

Since both ddC and EtB were ineffective in reducing mtDNA content in cultured cortical neurons, we hypothesized that differentiated central neurons have an extremely slow rate of mtDNA turnover. To test this hypothesis, we used the cell line P 19. P 19 cells are derived from a mouse embryonic carcinoma and grow rapidly in the undifferentiated state, but can be differentiated into neuronal cells by treatment with retinoic acid and growth on a glial feeder layer [14]. Thus, we could study the effects of the mtDNA synthesis inhibitor EtB on the mouse cell line before and after differentiation. Differentiated P 19 cells express both *N*-methyl-D-aspartate (NMDA) and non-NMDA type glutamate receptors and are susceptible to glutamate excitotoxicity, a feature unique to central neurons. Treatment of undifferentiated P 19 cells with 0.25 μg/mL EtB for 4 days reduced the mtDNA content in these cells by 74 ± 6% (Fig. 3A, lane 2, and Fig. 3B). We then tested the effect of EtB on differentiated P 19 cells. Four-day treatment with 0.25 μg/mL EtB did not affect mtDNA content significantly in differentiated P 19 neurons (Fig. 3A, lanes 3 and 4, and Fig. 3B). Thus, after differentiation, P 19 cells were no longer sensitive to EtB.

To induce differentiation, P 19 cells were plated onto a confluent monolayer of rat glial cells. Because mtDNA from rat and mouse is polymorphic, the mouse mtDNA from differentiated P 19 cells (the middle two bands in Fig. 3A, lanes 3 and 4) can be distinguished from rat mtDNA from the glial cells (the top and bottom bands). We analyzed the glial mtDNA in these experiments as well, and found no significant difference between control and EtB-treated glial cells (total DNA from EtB-treated relative to control cells was 92 ± 3%; mtDNA from EtB-treated relative to control cells was 103 ± 13%). Thus, glial cells in our cultures were also insensitive to EtB under these experimental conditions. The differentiated P 19 cultures were treated with 20 μM AraC to inhibit growth of both the glial feeder layer as well as any undifferentiated P 19 cells. It is possible that the arrested growth of the glial cells accounts for the insensitivity of the rat mtDNA to EtB.

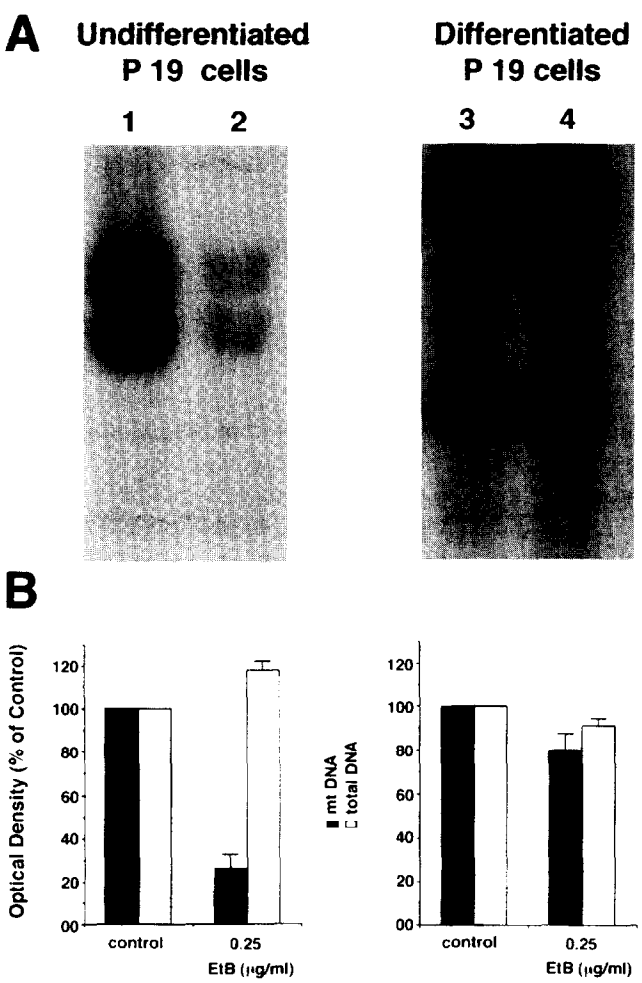

FIG. 3. Reduction in mtDNA content in undifferentiated P 19 cells but not in P 19 cells differentiated into a neuronal phenotype by EtB. (A) Hybridization of mouse mtDNA probe to *Bam*HI fragments of total cellular DNA prepared from control and EtB-treated cells. Lanes: 1 and 2, undifferentiated P 19 cells; 1, control; 2, 0.25 μg/mL EtB; 3 and 4, differentiated P 19 cells; 3, control; 4, 0.25 μg/mL EtB. (B) Quantitative analysis of total DNA and mtDNA in each sample of EtB-treated and control cells. Total DNA (open bars) and mtDNA (solid bars) were quantified by scanning the ethidium-stained gels and the autoradiograms, respectively with a densitometer. Data represent means ± SEM from at least three experiments.

DISCUSSION

In this study, we have shown that ddC, an inhibitor of DNA polymerase γ, effectively reduced mtDNA content in CEM cells, but not in cultured rat cortical neurons. We were concerned about the efficacy of ddC in different cell types. Therefore, we applied another mtDNA synthesis inhibitor, EtB, to these cells. EtB intercalates into DNA; thus, it has a generalized inhibitory effect on mtDNA synthesis in a broad range of cell types [7]. Consistent with this hypothesis, we have shown that EtB is effective in reducing mtDNA levels in both CEM cells and undifferentiated P 19 cells. However, EtB did not have a significant effect on mtDNA levels in rat cortical neurons. Lastly, although EtB reduced the mtDNA content in undifferentiated P 19 cells the same treatment with EtB was ineffective after P 19 cells were differentiated into neuronal cells. Thus, it is the differentiated neuronal status that makes cultured rat cortical neurons and differentiated P 19 neurons insensitive to mtDNA synthesis inhibitors.

mtDNA is in a dynamic balance between continued synthesis and degradation. Since mtDNA synthesis inhibitors only inhibit replication, a decrease in mtDNA content

in response to these drugs indicates a high turnover rate for mtDNA [12]. mtDNA synthesis in mammalian cells has been shown previously to be continuous, and the mtDNA turnover rate has been thought to increase with the degree of differentiation [5, 17]. Given that ddC depleted mtDNA content in both differentiated PC 12 cells [6] and cultured rat dorsal root ganglion cells [10], it was surprising that both ddC and EtB were ineffective in reducing mtDNA content in cultured cortical neurons and differentiated P 19 cells. Differentiated P 19 cells are similar to central neurons in that they express glutamate receptors, whereas differentiated PC 12 cells are similar to sympathetic neurons. It is possible that central neurons and peripheral neurons are fundamentally different in the rate at which mtDNA is replicated, the former having a much slower turnover of mtDNA. Consistent with this hypothesis, treatment of rabbits with ddC resulted in neurological and electrophysiological deficits in peripheral nerves with myelin, and axonal pathology, but had no effects in the CNS [18, 19]. Furthermore, prolonged treatment of AIDS patients with ddC often leads to reversible peripheral neuropathy, a degeneration of sensory and motor nerves [20, 21]. An early study showed that mtDNA in the rat brain turned over with a half-life of approximately 30 days, a rate 3- to 5-fold slower than that in peripheral tissues [22]. Thus, an insensitivity of cultured rat cortical neurons to mtDNA synthesis inhibitors suggests that the turnover of mtDNA in cultured central neurons is also very slow.

The fact that many transformed cell lines, including CEM and undifferentiated P 19 cells, are very sensitive to mtDNA synthesis inhibitors, but normal neurons and glia are relatively insensitive suggests that neuroblastomas and gliomas might be selectively sensitive to these drugs. Intrathecal administration of drugs that target mtDNA replication may provide a new strategy to inhibit brain tumor growth while sparing differentiated cells, although human cell lines devoid of mtDNA have the ability to form tumors *in vivo* [23].

Because DNA replication introduces errors and mtDNA exhibits a high rate of mutations [9], a slow turnover of mtDNA in neurons might protect the CNS, because a slow rate of replicative activity would reduce the chance of accumulating mtDNA mutations. This appears to be contradicted by a high rate of phenotypic expression of mitochondrial diseases in the CNS. However, the preferential expression of mitochondrial diseases in the CNS is probably more dependent on the postmitotic nature and the high energy demand of neuronal cells [1]. Increased synthesis of mtDNA has been reported in response to mtDNA damage [24]. Exposure to methyl mercury, a neurotoxin, induced an increase in mtDNA synthesis in cat neural tissues [17]. It would be interesting to determine whether the replication of mtDNA in patients with mitochondrial diseases was increased.

In conclusion, we have demonstrated that cultured rat cortical neurons are less sensitive to mtDNA synthesis inhibitors than cell lines, suggesting that the turnover of mtDNA in these cells is relatively slow. A slow replication of mtDNA in central neurons might benefit the central nervous system, although this may change in certain disease states.

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References

1. Wallace D, Mitochondrial DNA mutations in diseases of energy metabolism. *J Bioenerg Biomembr* **26:** 241–273, 1994.
2. Beal MF, Hyman BT and Koroshetz W, Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases? *Trends Neurosci* **16:** 125–131, 1993.
3. Wallace DC, Mitochondrial genetics: A paradigm for aging and degenerative diseases? *Science* **256:** 628–632, 1992.
4. Cooper JM and Clark JB, The structural organization of the mitochondrial respiratory chain. In: *Mitochondrial Disorders in Neurology* (Ed. Schapira AHV), pp. 1–29. Butterworth-Heinemann, New York, 1994.
5. Clayton DA, Replication of animal mitochondrial DNA. *Cell* **28:** 693–705, 1982.
6. Chen C-H, Vazquez-Padua M and Cheng Y-C, Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity. *Mol Pharmacol* **39:** 625–628, 1991.
7. Desjardins P, Frost E and Morais R, Ethidium bromide-induced loss of mitochondrial DNA from primary chicken embryo fibroblasts. *Mol Cell Biol* **5:** 1163–1169, 1985.
8. King M and Attardi G, Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* **246:** 500–503, 1989.
9. Schapira AHV, Mitochondrial cytopathies. *Curr Opin Neurobiol* **3:** 760–767, 1993.
10. Werth JL, Zhou B, Nutter LM and Thayer SA, 2′,3′-Dideoxycytidine alters calcium buffering in cultured dorsal root ganglion neurons. *Mol Pharmacol* **45:** 1119–1124, 1994.
11. Lewis W, Gonzalez B, Chomyn A and Papoian T, Zidovudine induces molecular, biochemical, and ultrastructural changes in rat skeletal muscle mitochondria. *J Clin Invest* **89:** 1354–1360, 1992.
12. Parker W and Cheng YC, Disruption of energy metabolism and mitochondrial function. In: *Neurotoxicology: Approaches and Methods* (Eds. Chang LW and Slikker WJ), pp. 483–490. Academic Press, San Diego, 1994.
13. Thayer SA, Murphy SN and Miller RJ, Widespread distribution of dihydropyridine-sensitive calcium channels in the central nervous system. *Mol Pharmacol* **30:** 505–509, 1986.
14. Turetsky DM, Huettner JE, Gottlieb DI, Goldberg MP and Choi DW, Glutamate receptor-mediated currents and toxicity in embryonal carcinoma cells. *J Neurobiol* **24:** 1157–1169, 1993.
15. Gadaleta G, Pepe G, De Candia G, Quagliariello C, Sbisa E and Saccone C, The complete nucleotide sequence of the *Rattus norvegicus* mitochondrial genome: Cryptic signals revealed by comparative analysis between vertebrates. *J Mol Evol* **28:** 497–516, 1989.
16. Nass MM, Differential effects of ethidium bromide on mitochondrial and nuclear DNA synthesis *in vivo* in cultured mammalian cells. *Exp Cell Res* **72:** 211–222, 1972.
17. Miller CT, Krewski D and Tryphonas L, Methylmercury-induced mitochondrial DNA synthesis in neural tissue of cats. *Fundam Appl Toxicol* **5:** 251–264, 1985.
18. Anderson TD, Davidovich A, Arceo R, Brosnan C, Arezzo J

and Schaumburg H, Peripheral neuropathy induced by 2',3'-dideoxycytidine. A rabbit model of 2',3'-dideoxycytidine neurotoxicity. *Lab Invest* **66:** 63–74, 1991.
19. Feldman D, Brosnan C and Anderson TD, Ultrastructure of peripheral neuropathy induced in rabbits by 2',3'-dideoxycytidine. *Lab Invest* **66:** 75–85, 1991.
20. Dubinsky R, Yarchoan R, Dalakas M and Broder S, Reversible axonal neuropathy from the treatment of AIDS and related disorders with 2',3'-dideoxycytidine (ddC). *Muscle Nerve* **12:** 856–860, 1989.
21. Yarchoan R, Thomas R, Allain J, McAtee N, Dubinsky R and Mitsuya H, Phase I studies of 2',3'-dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). *Lancet* **i:** 76–81, 1988.
22. Gross NJ, Getz GS and Rabinowitz M, Apparent turnover of mitochondrial deoxyribonucleic acid and mitochondrial phospholipids in the tissues of the rat. *J Biol Chem* **244:** 1552–1562, 1969.
23. Morais R, Zinkewich PK, Parent M, Wang H, Babai F and Zollinger M, Tumor-forming ability in athymic nude mice of human cell lines devoid of mitochondrial DNA. *Cancer Res* **54:** 3889–3896, 1994.
24. Miyaki M, Yatagai K and Ono T, Strand breaks of mammalian mitochondrial DNA induced by carcinogens. *Chem Biol Interact* **55:** 321–329, 1977.