Differential Effect of Ethidium Bromide and Cytosine Arabinoside on Mitochondrial and Nuclear DNA Synthesis in HeLa Cells

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The effect of ethidium bromide (EB) on the synthesis of circular DNA of mammalian cells was studied by isopycnic centrifugation in a CsCl-EB solution. EB (0.1–0.5 $\mu\text{g/ml}$) interferes with the synthesis of newly-formed circular DNA of HeLa cell mitochondria and causes degradation of the pre-existing circular DNA, as well. Under the same conditions, nuclear DNA synthesis was not inhibited. This effect was not reversible at a concentration of 0.5 μg EB/ml or more. Cytosine arabinoside (ara-C) did not exhibit an effect similar to that of EB.

Recently we have observed that ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide) (EB) at concentrations of 0.1 $\mu\text{g/ml}$ or more inhibits the incorporation of ^3H -thymidine into the circular double-stranded DNA of mitochondria in HeLa cells¹. The question arose whether EB only inhibits the synthesis of the newly-formed circular DNA of mitochondria or also degrades pre-existing circular DNA. The reversibility of the EB effect was another point to be clarified. Of importance also was a comparison of the effect of EB with that of cytosine-arabinoside (ara-C).

Materials and Methods

Cells: M HeLa cells were grown as monolayer cultures in double-strength Eagle's Basal Medium (2X BME) supplemented with 10% fetal calf serum (FCS).

Chemicals: EB was purchased from Calbiochem, Los Angeles, Calif., ^3H -thymidine (specific radioactivity, 20 Ci/mmol) and ^{14}C -thymidine (specific radioactivity, 24.5 mCi/mmol), from New England Nuclear, Boston, Mass., and cytosine-arabinoside from Sigma Chemical Company, Missouri.

Extraction of mitochondrial DNA, centrifugation conditions, and measurement of radioactivity: Crude preparations of mitochondrial DNA of HeLa cells were obtained by the extraction method using 1% sodium dodecyl sulfate (SDS) and 100 μg predigested pronase per ml and by the fractionation procedure described by HIRT³. Isopycnic centrifugation of the supernatant was performed in a CsCl solution containing 100 μg EB/ml (mean density, 1.550) for 24 h at 43,000 rev/min at 20° (see ref.³) using at 50 fixed-angle rotor in a Beckman Model L₂ centrifuge. Nuclear DNA was extracted as described previously⁵.

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The fractions were collected from the bottom of the gradients and precipitated on millipore filters (0.45 μ) with cold trichloroacetic acid as described previously¹. The radioactivity was measured in a Beckman liquid scintillation counter using a toluene – Liquiflour (New England Nuclear) mixture.

Results

Effect of EB on the circular DNA of HeLa cell mitochondria: Previous experiments showed that EB inhibits the synthesis of newly-formed circular mitochondrial DNA in HeLa cells¹. To examine whether it also acts on pre-existing circular DNA, we labeled approximately 5×10^6 HeLa cells with ^{14}C -thymidine (1.0 $\mu\text{Ci/ml}$) for 48 h. The medium was then changed, and the cells were exposed to ^3H -thymidine (5 $\mu\text{Ci/ml}$) and EB (0.5 $\mu\text{g/ml}$) for an additional 48 h. No EB was added to the control culture. After a total incubation period of 96 h, the mitochondrial DNA was extracted by the SDS-pronase method described by HIRT³.

If EB interfered only with the synthesis of newly-formed circular DNA, ^{14}C -labeled, covalently-closed, circular, double-stranded DNA would be detectable by isopycnic centrifugation in a CsCl-EB solution. If, however, EB also degraded pre-existing circular DNA, no radioactivity should occur in the position of double-stranded circular DNA.

In the control samples, a clear radioactivity peak was obtained for ^3H and ^{14}C in the region of the circular mitochondrial DNA (Fig. 1 a), whereas in the EB-treated samples no specific radioactivity peak was exhibited in this region (Fig. 1 b).

The total ^3H -radioactivity of circular DNA region in the EB-treated sample was 3.2% of that in the

Fig. 1. Degradation of pre-existing mitochondrial DNA of HeLa cells by EB. Isopycnic centrifugation in a CsCl-EB solution was performed on a crude preparation of mitochondrial DNA, (a) extracted from 5×10^6 HeLa cells labeled for 48 h with ^{14}C -thymidine ($1.0 \mu\text{C}/\text{ml}$) ($\bullet\text{---}\bullet$), and for another 48 h with ^3H -thymidine ($5 \mu\text{C}/\text{ml}$) ($\circ\cdots\circ$); b) extracted from 5×10^6 HeLa cells labeled with ^{14}C -thymidine ($1.0 \mu\text{C}/\text{ml}$) for 48 h and exposed to ^3H -thymidine ($5 \mu\text{C}/\text{ml}$) and EB ($0.5 \mu\text{g}/\text{ml}$) for the following 48 h. The lighter fraction of linear double stranded DNA represents nuclear DNA fragments contaminating the crude preparation of mitochondrial DNA after SDS-pronase extraction from HeLa cells according to HIRT³. Centrifugation conditions: CsCl-EB ($100 \mu\text{g}/\text{ml}$); mean density, 1.55; 50 fixed-angle rotor; 43,000 rev/min; 24 h; 20°C . The buoyant densities ($\bullet\text{---}\bullet$) of circular and linear DNA are 1.607 and $1.553 \sim 1.5555 \text{ g}/\text{cm}^3$, respectively.

Treatment of cells [μg EB/ml for 48 h]	Total radioactivity (^3H) of circular DNA fraction	
	net counts/min	% control
0.5	669	3.2
Control	27,076	100

Table 1. Inhibition of Synthesis of Closed-Circular Mitochondrial DNA in HeLa Cells by Ethidium Bromide.

Total ^3H -radioactivity of circular double-stranded DNA is calculated from CsCl-EB gradient shown in Fig. 1.

control, and for ^{14}C -radioactivity, 8.2% (Tables 1 and 2). This result indicated that EB not only inhibits newly-synthesized circular DNA, but also degrades pre-existing circular DNA.

Treatment of cells [μg EB/ml for 48 h]	Total radioactivity (^{14}C) of circular DNA fraction	
	net counts/min	% control
0.5	26	8.2
Control	316	100

Table 2. Degradation of Pre-Existing Closed-Circular Mitochondrial DNA in HeLa Cells by Ethidium Bromide.

Total ^{14}C -radioactivity of circular double-stranded DNA is calculated from CsCl-EB gradient shown in Fig. 1.

Reversibility of the effect of EB: To examine whether the effect of EB is reversible, we exposed 5×10^6 HeLa cells to concentrations of 0.1 or $0.5 \mu\text{g}$ EB/ml of culture medium for 48 h. The medium was then changed and the cells were continuously labeled with ^3H -thymidine ($5 \mu\text{C}/\text{ml}$) for an additional 48 h. The mitochondrial DNA was then extracted by the SDS-pronase method of HIRT³ and analyzed by isopycnic centrifugation in a CsCl-EB solution.

Limited radioactivity was obtained in the position of circular double-stranded DNA. In this case the total radioactivity for the circular DNA region in the EB-treated samples was 22.4% ($0.1 \mu\text{g}$ EB/ml) and 9.6% ($0.5 \mu\text{g}$ EB/ml) of the control (Table 3).

Treatment of cells [μg EB/ml for 48 h]	Total radioactivity (^3H) of circular DNA fraction	
	net counts/min	% control
0.1	4,723	22.4
0.5	2,016	9.6
Control	21,076	100

Table 3. Reversibility of the Effect of Ethidium Bromide.

Total radioactivity of circular double-stranded DNA is calculated from CsCl-EB gradient (not given here). Mitochondrial DNA was extracted from HeLa cells after exposure to 0.1 or $0.5 \mu\text{g}$ EB/ml of culture medium for 48 h. The medium was then changed and the cells were exposed to ^3H -thymidine ($5 \mu\text{C}/\text{ml}$) for the following 48 h.

Comparison of the effect of EB with that of cytosine-arabinside: To ascertain the specificity of the action of EB, the effect of cytosine-arabinside was examined in the same system. The compound was added simultaneously with ^3H -thymidine ($5 \mu\text{C}/\text{ml}$) to the culture medium of approximately 5×10^6 HeLa cell monolayer cultures. After 48 h, the mitochondrial DNA was extracted and analyzed by isopycnic centrifugation in a CsCl-EB solution.

As shown in Fig. 2, at the chosen concentrations cytosine-arabinside ($10 \mu\text{g}/\text{ml}$) did not exhibit an effect similar to that of EB. In contrast to EB, which

Fig. 2. Effect of EB and cytosine-arabioside on the synthesis of mitochondrial DNA in HeLa cells. Isopycnic centrifugation in an CsCl-EB solution was performed on mitochondrial DNA, (a) extracted from 5×10^6 HeLa cells labeled with ^3H -thymidine ($5 \mu\text{C}/\text{ml}$) ($\circ-\circ$) for 48 h; (b) extracted from 5×10^6 HeLa cells labeled with ^3H -thymidine for 48 h and simultaneously exposed to $0.5 \mu\text{g}$ EB/ ml ($\circ-\circ$); (c) extracted from 5×10^6 HeLa cells labeled with ^3H -thymidine ($5 \mu\text{C}/\text{ml}$) for 48 h and simultaneously exposed to cytosine-arabioside ($10 \mu\text{g}/\text{ml}$) ($\circ-\circ$). Centrifugation conditions: CsCl-EB ($100 \mu\text{g}/\text{ml}$); mean density 1.55; 50 fixed-angle rotor; 43,000 rev/min; 24 h; 20°C . The buoyant densities ($\bullet-\bullet$) of circular DNA are 1.607 (a) and 1.606 (b), of linear DNA 1.564 (a), 1.570 (b), 1.568 (c). The linear DNA fraction represents contaminating nuclear DNA fragments (see legend Fig. 1).

strongly inhibits the synthesis of mitochondrial DNA, but does not affect that of nuclear DNA, cytosine-arabioside strongly inhibited the nuclear DNA synthesis (Table 4) and slightly the mitochondrial DNA synthesis (Fig. 2 c). In Fig. 2 c, most of the radioactivity in the region of linear DNA ($\rho = 1.568$) may be explained by the observation that cytosine-arabioside causes a large amount of degradation of nuclear DNA (unpublished data).

Treatment of cells [$\mu\text{g}/\text{ml}$ for 48 h]	Specific radioactivity of nuclear DNA as % control	
Ethidium bromide	1.0	94
	0.5	116
Cytosine-arabioside	10	1
	1	14
Control		100 *

Table 4. Comparison of the Effects of EB and Cytosine-Arabioside on the Synthesis of Nuclear DNA in HeLa Cells.

* Mean value, 2196 counts/min $^3\text{H}/\mu\text{g}$ DNA.

Specific radioactivity (cpm/ μg DNA) of nuclear DNA of HeLa cells labeled with ^3H -thymidine ($5 \mu\text{C}/\text{ml}$) for 48 h and simultaneously treated with EB or cytosine-arabioside was determined.

Discussion

Our results show that EB interferes differentially with the synthesis of closed circular double-stranded DNA of mitochondria in HeLa cells. It also degrades preexisting circular DNA. These effects are not reversible during the time period observed.

The action of EB, therefore, cannot be explained by the specific inhibition of the mitochondrial DNA polymerase alone⁶. Its effect may also be the consequence of a degradation of the circular DNA, caused by direct interaction between DNA and EB⁴. The degradation might furthermore result from an indirect action of EB on the circular DNA by the inactivation of repair enzymes or activation of endonucleases. Further experiments are needed to elucidate this result.

In any case differential inhibition of the synthesis of circular DNA of mitochondria with EB could be a useful tool in studying the as-yet-unknown relation between nuclear- and mitochondrial-DNA-directed apparatus of the cell. Investigations in this direction are in progress.

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¹ K. RADSACK, K. KATO, Y. SATO, and H. KOPROWSKI, Exp. Cell Res. **66**, 410 [1971].

² L. HAYFLICK, Exp. Cell Res. **37**, 614 [1965].

³ B. HIRT, J. molecular Biol. **26**, 365 [1967].

⁴ R. RADLOFF, W. BAUER, and J. VINOGRAD, Proc. nat. Acad. Sci. USA **57**, 1514 [1967].

⁵ K. BERNS and C. A. THOMAS, JR., J. molecular Biol. **11**, 476 [1965].

⁶ R. R. MEYER and M. V. SIMPSON, Biochem. biophys. Res. Commun. **34**, 238 [1969].