

Inhibition of Phytohaemagglutinin - Induced Lymphocyte Transformation and Mitosis by Ethidium Bromide

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Abstract

The effect of ethidium bromide (EB) on phytohaemagglutinin (PHA) induced lymphocyte transformation and mitosis was evaluated by morphology and ³H-thymidine uptake. EB at low concentrations is known to specifically bind to mitochondrial DNA and selectively inhibit mitochondrial protein synthesis. However at these concentrations EB inhibited both transformation and mitosis. Electron micrographs of lymphocytes cultured with PHA showed mitochondria of normal structure with round, oval or elongated shapes, well-defined cristae and electron dense matrix. However micrographs of PHA stimulated lymphocytes cultured with EB showed swollen mitochondria with fewer cristae and vacuolation of the matrix. Thus damage to mitochondria by EB provides structural evidence of inhibited mitochondrial protein synthesis. This study shows that EB by blocking mitochondrial protein synthesis inhibits the nuclear DNA synthesis and division of proliferating lymphocytes. These observations suggest that mitochondrial protein synthesis is crucial for cell proliferation (JPMA 32:114, 1982).

Introduction

Ethidium bromide (EB) is a fluorescent dye which belongs to the class of phenanthridinium drugs. It binds to DNA by intercalating between adjacent base pairs of DNA double helix (Waring, 1965). EB has a low affinity for deoxynucleo-protein (DNP) as compared to purified DNA (Angerer and Moudrianakis, 1972). This high affinity for pure DNA results in the specific inhibitory effects of EB on mitochondrial DNA in mammalian cells.

EB at low concentrations 0.1-5.0 ug/ml has been shown to inhibit selectively (a) mitochondrial associated RNA synthesis with no effects on RNA occurring outside the mitochondria (Zylber et al., 1969), (b) the activity of mitochondrial DNA polymerase (Meyer and Simpson, 1969), (c) mitochondrial DNA synthesis while nuclear DNA synthesis was unaffected or stimulated (Nass, 1972) and (d) mitochondrial protein synthesis (Lenk and Penman, 1971; Perlman and Penman, 1970) while cytoplasmic protein synthesis is unaffected (Avadhan and Rutman, 1975). Ultrastructural studies on mammalian cells treated with ethidium bromide have shown damage to mitochondria, with reduced number of cristae and swelling and these effects have been thought to result from the inhibition of mitochondrial protein synthesis (Lenk and Penman, 1971; McGill et al., 1973).

To study the role of mitochondrial protein synthesis in cell proliferation, we have investigated the effects of low concentrations of EB 0.1-3.0 ug/ml on transformation and mitosis of human peripheral blood lymphocytes stimulated by phytohaemagglutinin (PHA). We have also observed the effect of EB on the mitochondrial ultrastructure of these cells.

Material and Methods

Peripheral blood lymphocytes of normal individuals were separated on Lymphoprep (Nyegaard). All cultures were set up with 3×10^6 cells in 3 ml TC-199 (Wellcome) with 20% fetal calf serum (FCS) (Gibco-Biocult). 0.1 ml of PHA diluted 1:3 with medium was added to the control and the test cultures.

Ethidium bromide (Sigma) at concentrations of 0.1, 0.5, 1, 2 and 3 µg/ml was added just before the cultures were incubated. Incubation was at 37°C in a humid atmosphere and the cultures were harvested after 72 hours and 96 hours. All cultures were performed in duplicate. Transformation and mitosis were assessed by morphology on cytocentrifuge slides prepared with 0.5×10^6 cells and stained with May-Grunwald-Giemsa. The percentage of transformed and mitotic cells was scored by counting 1000 cells on slides. Transformation was also assessed by ^3H -thymidine incorporation. ^3H -thymidine 40 Ci/mmol (Amersham) at a concentration of 1 µCi/3 ml of culture was added 4 hours before harvesting. The cells were washed once in PBS, twice with 5% trichloroacetic acid (TCA) and once with methanol. The cell pellet was dissolved in 0.2 ml of 0.1M sodium hydroxide and kept at 56°C for 45 minutes. Two ml of methanol was added followed by 10 ml of a toluene-based scintillant with a 2:1 ratio of toluene and triton-X100, 0.4% PPO and 0.01% POPOP. After light and temperature stabilisation the samples were counted in an Intertechnique SL-30 liquid scintillation counter. To study the possibility of a non-specific toxic effect of this drug, lymphocytes were incubated at 37°C in the presence of ethidium bromide at 1 and 3 µg/ml in culture medium for 96 hours. The cells were washed twice in medium and viability assessed by counting the percentage of cells unstained with Trypan Blue. Finally the cells were cultured with PHA for 72 hours and transformation assessed by ^3H -thymidine uptake. Lymphocytes from the cultures were fixed in 3% glutaraldehyde in 0.1M phosphate buffer pH 7.0 for 2 hours at 20°C and washed three times in 0.1M phosphate buffer containing 0.1M sucrose. They were then post-fixed in 1% osmium tetroxide in Millonig's buffer for 1 hour at 4°C and washed twice in distilled water. Samples were pre-embedded in 3% agar, dehydrated through a series of graded alcohols, taken through propylene oxide and finally embedded in araldite. Sections were cut with an LKB III ultramicrotome, stained with alcoholic uranyl acetate and Reynolds lead citrate. An AEI 6B electron microscope was used to view the sections.

Result

Transformation assessed by ^3H -thymidine uptake:

Results are expressed as percentage of controls with PHA added but no EB. Ethidium bromide showed a marked inhibitory effect on thymidine uptake (Fig. 1).

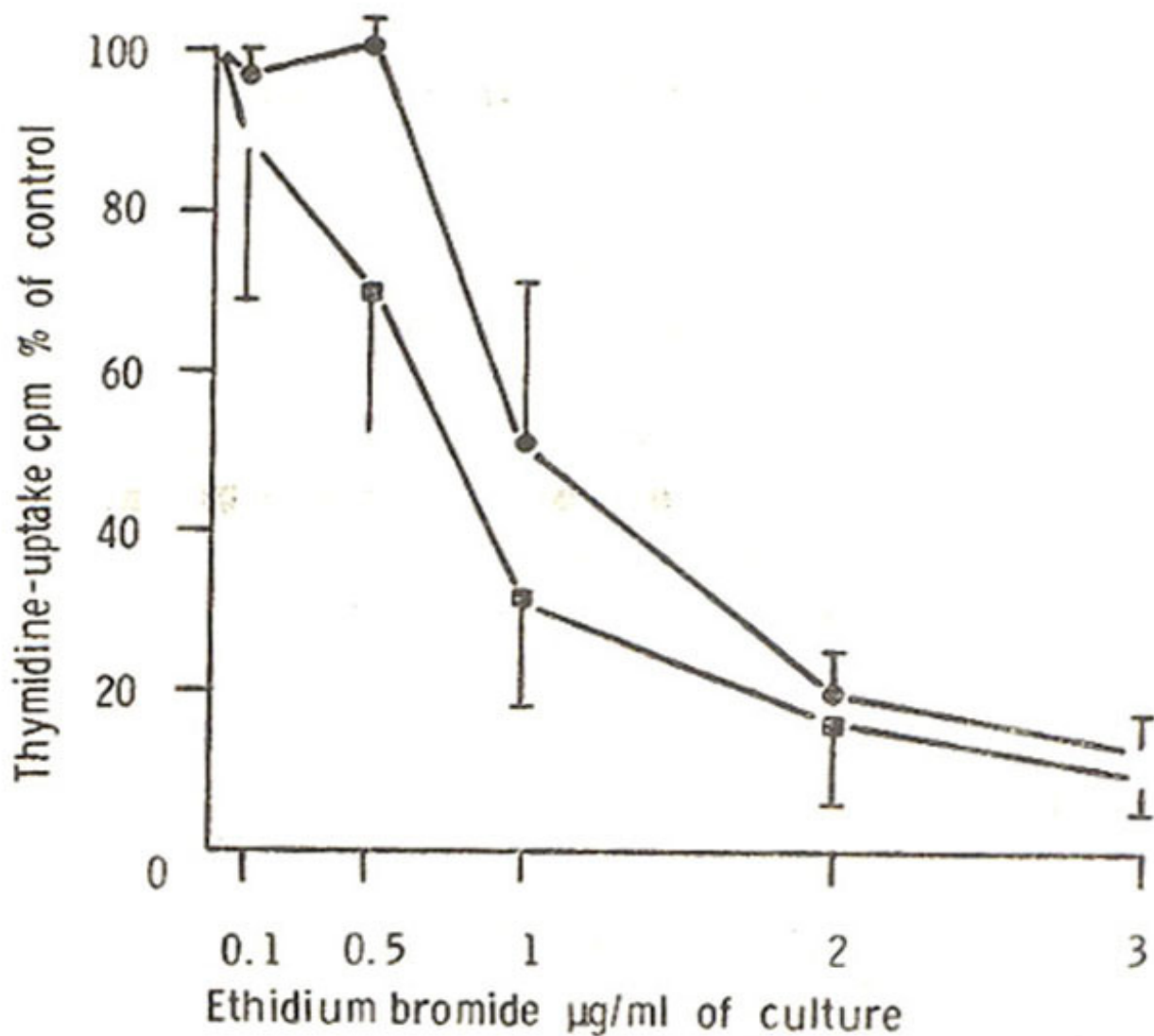


Fig. 1: Effect of ethidium bromide on ^3H -thymidine uptake of PHA-stimulated lymphocyte cultures. Results are expressed as percentage of control cultures with PHA but without drug. Cultures were harvested at Day 3 ●—● and Day 4 ■—■. Each value is a mean of six independent experiments and bars show standard deviation.

A dose dependent inhibition was observed with a maximum effect on Day 4. No inhibition was observed with 0.1 µg/ml or 0.5 µg/ml at Day 3, but at the latter concentration there was 30% inhibition on Day 4. At 1 µg/ml on Day 3 there was 49.5% inhibition whilst an almost complete inhibition was observed with higher concentrations.

Transformation and mitosis assessed by morphological studies:

Normal lymphocytes synthesize DNA and divide following PHA stimulation. Transformation after exposure to ethidium bromide was expressed as a percentage of the control value where PHA alone was present. Ethidium bromide at low concentrations had little effect on transformation though mitosis was inhibited while higher concentrations inhibited both on Day 3 of culture (Fig. 2 and Table 1).

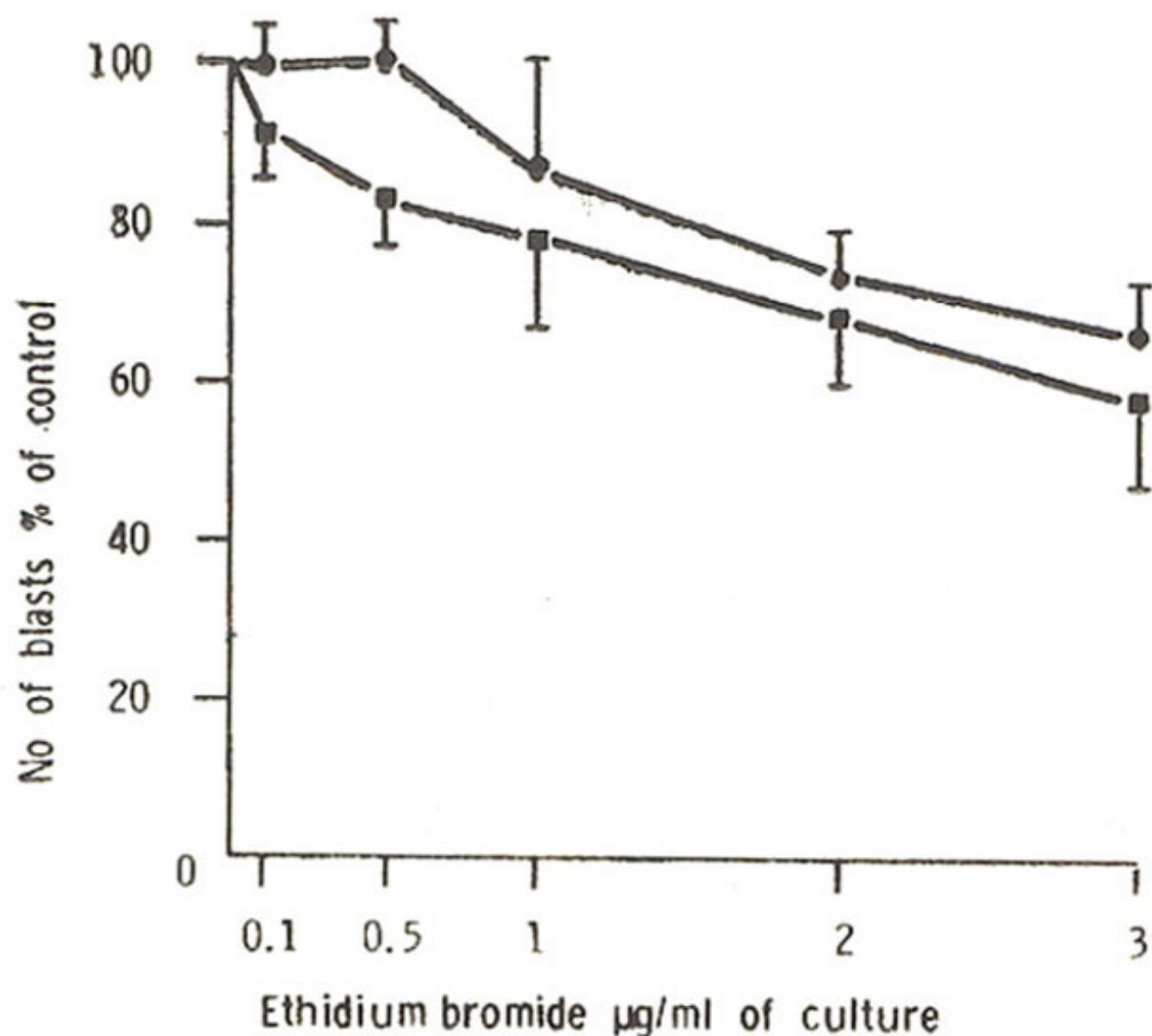


Fig. 2: Effect of ethidium bromide on blastic transformation of PHA-stimulated lymphocytes in culture. Results are percentage of transformed cells on slides expressed as percentage of controls with PHA but without drug. Cultures were harvested at Day 3 ●---● and Day 4 ■---■. Each value is a mean of six independent experiments and bars show standard deviation.

Table I
Inhibition of Mitosis in PHA-stimulated Lymphocytes by Ethidium Bromide (EB)

<i>Culture/ ug/ml of drug</i>	<i>Mitotic index % Day 3</i>	<i>% ± SD Day 4</i>
PHA	3.2 ± 0.75	4.0 ± 0.5
PHA + 0.1 EB	3.1 ± 0.35	2.0 ± 1.0
PHA + 0.5 EB	2.7 ± 0.35	1.0 ± 0.5
PHA + 1 EB	1.6 ± 1.15	1.5 ± 0.7
PHA + 2 EB	0.2 ± 0.35	0.5 ± 0.7
PHA + 3 EB	0.0	0.5 ± 0.7

On Day 4 of culture all concentrations moderately inhibited transformation (Fig. 2) and a marked inhibition of mitosis was observed (Table I). With higher concentration of ethidium bromide of 3 ug/ml some disrupted cells were observed on slides.

Lymphocytes were cultured with PHA, with and without the drugs for 3 and 4 days. The percentage of mitotic cells was assessed by counting 1000 cells. Values represent mean of six different experiments with standard deviations.

Viability tests:

Ethidium bromide had little or no effect on the viability of unstimulated lymphocytes incubated with each drug for 96 hours when compared to controls incubated without EB. The subsequent response to PHA stimulation was similar in the control cultures and in those to which the drugs were added, except for higher concentration of ethidium bromide (Table 2).

Table II

Effect of Ethidium Bromide (EB) on Viability of Unstimulated Lymphocytes and their Subsequent Transformation.

<i>Drug concentration ug/ml</i>	<i>Viability : % of cells unstained with Trypan Blue after 4 days incubation</i>	<i>³H-thymidine uptake at 72 hours (cpm)</i>
Control	94.2 ± 1.1	11940 ± 1828
+ 1.0 (EB)	91.5 ± 2.1	13302 ± 2779.6
+ 3.0 (EB)	86.3 ± 8.8	9532 ± 1272

Lymphocytes were incubated with and without drug for 4 days in culture medium with 20% (FCS). Cells were washed twice with medium and their viability assessed by Trypan Blue extrusion. These cells were subsequently cultured with PHA for 3 days and transformation assessed by ³H-thymidine uptake. Each value represents the mean of three independent experiments with standard deviations. Mitochondrial ultrastructure in cultured lymphocytes :



Plate 1: PHA-transformed lymphocyte from a control culture without drug. Mitochondria of various shapes and sizes with well defined cristae are observed. Magnification x 30,000.



Plate 2: PHA-transformed lymphocyte from a culture with 1 ug/ml ethidium bromide. Mitochondria are swollen with reduced number of cristae. Magnification x 20,000.

Electron micrographs of PHA-transformed lymphocytes at day 4 without EB (3 experiments) showed mitochondria of oval, round and elongated shapes. 90 to 95% of the blasts had mitochondria with well-defined cristae and dense mitochondrial matrix (Plate 1). However, 70 to 85% of the PHA-transformed lymphocytes from cultures with ethidium bromide (3 experiments) had mitochondria with damaged cristae, together with varying degrees of vacuolation and swelling (Plate 2). Mitochondrial damage was more prominent with the higher concentration of the drug, where mitochondria without cristae and with extensive swelling were observed. Unstimulated lymphocytes from cultures with or without PHA and EB showed normal mitochondria. Structural damage was observed only in mitochondria and no other cell components were affected.

Discussion

Ethidium bromide (EB) inhibited PHA-stimulated lymphocytes and mitosis. The greater degree of inhibition of transformation observed in thymidine uptake studies, suggests that the cells may appear morphologically transformed but their DNA synthesis is markedly inhibited. Since mitosis was inhibited at 0.5 ug/ml with no effect on transformation at day 3, indicates that the cells were unable to complete their cell cycle. The increase in inhibition of transformation and mitosis on day 4 suggests a progressive loss of functional mitochondria as the cells go through the cycle, due to the inhibitory effects of the drug. This is confirmed by the fact that damaged mitochondria were observed only in cultures with EB.

Mitochondria synthesize a number of proteins, some of these constitute integral components of the respiratory chain cytochromes a + a³ (Weiss et al., 1974) and b (Weiss, 1972) and some are involved in cristae formation (Lenk and Penman, 1971). Thus the inhibition of mitochondrial protein synthesis results in the observed damage to mitochondria. The inhibitory effects of EB transformation and mitosis are probably related to the inhibition of mitochondrially synthesized components of the respiratory chain. This results in depression of respiratory activity, hence lowering of cellular ATP levels leading to the inhibition of cellular processes in these rapidly growing cells. Further more a low molecular weight-mitochondrially synthesized protein has been shown to be exported from the mitochondria to the cell membrane (Macklin et al., 1977). This finding and the inhibitory action of EB on viral induced transformation of mouse balb/ 3T3 cells (Roa and Bose, 1975) have led to the suggestion that mitochondrial proteins may play a part in control of cell transformation and division (Macklin et al., 1977). Therefore the transformation and mitosis inhibition observed here thus might be attributed firstly, to depression of respiratory activity and secondly to the inhibition of control proteins. Mitochondrial proteins may therefore be critical for transformation and division of proliferating cells. In fact drugs which block mitochondrial protein synthesis, inhibit actively proliferating cells as demonstrated by the *in vivo* antileukaemic effect of chloramphenicol (Oeeter et al., 1979) and the anti-tumour effect of EB (Nishiwaki et al., 1974). This inhibition of mitochondrial protein eventually leads to death of proliferating cells as shown with the higher concentration of EB in our study. A direct non-specific toxic effect of EB was excluded because at the concentrations used it had little or no effect on the viability of unstimulated lymphocytes nor did it effect the subsequent transformation of these cells. Further studies are in progress to elucidate the role of mitochondria in cell proliferation.

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