SV40-TRANSFORMED HAMSTER CELLS
RESISTANT TO 100-250 μg/ml OF
ETHIDIUM BROMIDE

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SUMMARY

SV40-transformed hamster cells were selected for resistance to ethidium bromide (EB). Several cell lines were established, which grew in the presence of up to 250 μg/ml EB. The EB resistance is genetically stable. The cloned resistant cells show no difference in morphology, with the exception of the mitochondrial ultrastructure, which exhibits condensed cristae formation. The tumorigenicity of these cells in Syrian gold hamsters is considerably reduced. Incorporation of radioactive labelled thymidine into mitochondrial DNA is not influenced by the presence of the drug. Gel electrophoresis with mitochondrial proteins from wild-type and resistant cells reveals significantly different patterns. The mechanism of EB resistance is discussed.

INTRODUCTION

Ethidium bromide (EB), a phenanthridine dye, intercalates in vitro and in vivo between adjacent base pairs of native double-stranded DNA (Crawford & Waring, 1967). In several eucaryotic cell systems the mitochondrial DNA is a prime target of this agent. Wild-type yeast can be converted by EB treatment to respiratory-deficient, cytoplasmically inherited petite phenotype mutants (Mahler, Mehrotra & Perlman, 1971). In mammalian cells EB causes highly selective effects on nucleic acids and protein synthesis within mitochondria (Upholt & Borst, 1974). Typically, mitochondria in EB-treated cells have a reduced cytochrome a-a₃ content, a variable reduction in the level of cytochrome b and abnormal morphology (Sato, Chance, Kato & Klietmann, 1973; Soslau & Nass, 1971; Stuchell, Weinstein & Beattie, 1975). EB-induced mitochondrial abnormalities are reversible. If EB is removed from the culture medium, apparently normal mitochondria are found in succeeding cell generations.

The exception are the petite mutations in yeast and several reported mutations in mammalian cell cultures. In 1973, Klietmann, Sato & Nass described a cell line of SV40-transformed hamster cells resistant to 16 μg/ml EB, and a HeLa cell line resistant to 1 μg/ml EB (Klietmann et al. 1973a). In 1974 Altaner & Matoska found and characterized an avian sarcoma virus-transformed hamster cell line resistant to EB.

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to 30 μg/ml EB. We were able to increase the 16 μg/ml resistance of the SV40-
transformed hamster cells derivate up to 250 μg/ml EB.

This communication describes some properties of these genetically stable mutants.

MATERIALS AND METHODS

Cell lines and tissue culture conditions

All cell lines were maintained and propagated in Eagle minimal essential medium (MEM),
supplemented with 10% calf serum (Seromed, Munich) and 100 μg/ml streptomycin and
100 I. U./ml penicillin. The medium containing EB (Calbiochem, Los Angeles, Calif.) was
prepared by addition of EB from a freshly made stock solution of 5 mg/ml.

The F5-1 cells were established by Girardi, Sweet & Hilleman (1963) and derived from
a primary tumour induced by inoculation of a newborn Syrian gold hamster (Lakeview
Hamster Colony, Newfield, N.J.) with SV40 virus.

SV40-transformed hamster cells resistant to 16 μg/ml EB (F16) were established by
Klietmann et al. (1973) from F5-1 cells. The presence of the SV40 specific T antigen in the
wild-type cell line and in the EB-resistant mutants was demonstrated by indirect immuno-
fluorescent tests, using sera of hamsters bearing SV40-induced tumours.

Cells used in this study were periodically tested by electron microscopy and found to be
free of mycoplasma.

Determination of cell growth rates

The method of cell growth rate determination was described by D’Agostino & Nass (1976).
The cells were grown in Petri dishes (60 cm²) at 37 °C in a humidified CO₂ incubator. At each
time point (see Fig. 1, p. 160) 4 plates of each cell type were harvested by trypsinization and
scraping with a rubber policeman. The cells were centrifuged at 300 g for 5 min, resuspended
in MEM, and counted with a haemocytometer.

Electron microscopy

The cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline for 2 h at
0 °C and postfixed for 1 h with 1% OsO₄ in PBS and embedded in Epon 812. Thin sections
were stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop IA
electron microscope.

Isolation of mitochondrial DNA from whole cells

Mitochondrial DNA was isolated by a modified Hirt (1967) procedure. The cells were
grown in roller bottles and labelled during the logarithmic phase of growth for 48 h with
4 μCi [Me-³H]thymidine/ml medium (50 ml medium/roller bottle). The [Me-³H]thymidine
(sp. act. 60-83 Ci/mmol) was purchased from New England Nuclear. The cells were harvested
by scraping with a rubber policeman, washed with PBS and sedimented by centrifugation.
The cells were resuspended in 3 vol. of DNA-buffer A (2 mM EDTA, 0.01 M Tris-HCl,
pH 7.5) and spread onto plastic Petri dishes (Smith, Jordan & Vinograd, 1971), 1% sodium
dodecyl sulphate was added, gently mixed and incubated at room temperature for 20 min.
The viscous layers were scraped into centrifuge tubes, CsCl was added to a final concentration
of 1-0 M, gently mixed and chilled to 0 °C overnight. This solution was centrifuged for 30 min
at 17,300 g. The density of the supernatant was adjusted with solid CsCl to 1.582 g/ml, ca.
350 μg/ml EB was added and 125 ml samples were centrifuged in a 50 Ti-rotor at 40,000 rev/
min for 48 h. The tubes were fractionated and aliquots of the fractions were applied to filter
paper disks. The samples were immersed in 3 changes of ice cold 5% trichloroacetic acid
supplemented with 1% tetraethylenediamine phosphate and 2 changes of ethanol for 10 min each,
dried and counted in a Tricarb Liquid-Scintillation counter.
Isolation of mitochondria

The radioactive labelled mitochondria were isolated according to the methods of Nass (1972). Approximately \( 1.5 \times 10^8 \) cells were scraped off with a rubber policeman, washed twice with PBS, resuspended in hypotonic medium (0.1 M sucrose, 2 mM EDTA, 0.025 M Tris-HCl pH 7.5) and disrupted in a Dounce homogenizer. The medium was made isotonic and the mitochondria were isolated by differential centrifugation. (Centrifugation at 800 g and 1000 g to remove nuclei and cell debris and at 10000 g to sediment mitochondria.) The mitochondrial pellet was layered onto a 11-ml linear sucrose gradient 1.03–1.1 M sucrose in 2 mM EDTA, 25 mM Tris-HCl (pH 7.3) and centrifuged for 50 min at 25000 rev/min in a Beckman SW 40 rotor at 4 °C. At equilibrium both the wild type and the EB-resistant mitochondria were found in a single band. The specific activities of cytochrome oxidase in the cell fractions were tested and the mitochondrial fraction was determined by its high specific activity of cytochrome oxidase.

Cytochrome assay

We assayed the cytochrome oxidase spectrophotometrically by following the oxidation of reduced cytochrome c as described by Wharton & Tzagoloff (1967). The protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Extraction of mitochondrial DNA from isolated mitochondria

The mitochondrial DNA was extracted from isolated mitochondria by the same methods described for isolation of mitochondrial DNA from whole cells.

Incorporation of thymidine into mitochondrial DNA from isolated mitochondria

Mitochondrial DNA of wild-type cells and cells resistant to 16 µg/ml EB was labelled in vitro according to Mitra & Bernstein (1970). The incubation mixture contained per ml, 15 nmol 3 H-dTTP (33 µCi; New England Nuclear). The amount of mitochondria assayed per ml was 500 µg. At appropriate intervals aliquots of 50 µl were removed from the reaction vessel, acid-precipitated on filter paper disks, and counted as described for the isolation of mitochondrial DNA from whole cells.

Gel electrophoresis

The method of electrophoresis and dissociation of mitochondrial proteins was based on the procedure described by White & Nandi (1976). All solutions used for isolation and purification of mitochondria contained 2 mM phenylmethylsulphonylfluoride. Samples of mitochondria (50 µg/ml protein) were disrupted by heating at 100 °C for 5 min with 5 M urea, 0.1 % SDS, and 2 % β-mercaptoethanol. The gels were composed of 10 % acrylamide, 0.27 % bisacrylamide in 4 M urea, 0.01 M EDTA, 0.1 % SDS, 0.1 % N,N,N',N'-tetramethylethylenediamine, and 0.05% of 1 M sodium phosphate buffer pH 7.2. Polymerization was catalysed with 0.5 % ammonium persulphate. The gels were electrophoresed at 5 mA/gel for 5 h in 0.05 M sodium phosphate buffer pH 7.2, containing 0.1 % SDS and 0.01 M EDTA. The gels were fixed, stained with Coomassie blue and destained with 75 % acetic acid + 5 % methanol. The gels were scanned with a gel scanner (Gilford). The molecular weights were determined by standards of tobacco mosaic virus (TMV) and bovine serum albumin (BSA).
RESULTS

Selection of EB-resistant cells

The ethidium bromide-resistant SV40-transformed hamster cells were established by cultivation of F-16 cells in EB-containing medium with increasing concentrations. We started with a concentration of 32 µg/ml EB. After 2 weeks of cultivation in this medium, when the cells were confluent and looked healthy, the EB concentration gradually was increased over 64, 100, 150, 200 to 250 µg/ml EB. This process was completed after a period of 5 months. Cell clones derived from a single cell were established out of well growing cultures. The medium was changed twice a week. The growth rates of mutants resistant to EB concentrations higher than 100 µg/ml were rather slow in the presence of the drug. Therefore we performed most of our characterization experiments with a cloned cell line, resistant to 100 µg/ml EB, designated 100 K.
Growth rates of wild type cells and mutants

Fig. 1 shows the growth rate of the wild-type cells F5-1 in normal medium in contrast to the growth in medium containing 100 μg/ml EB and the growth of 100K, the cells resistant to 100 μg/ml EB in EB-containing medium. The generation rate of the wild-type cells is ca. 14 h and of the mutants ca. 18 h. The wild-type cells died in EB-containing medium after an incubation time of 48 h.

Characterization of the mechanism of EB resistance

To decide whether the EB resistance of 100 K cells is genetically stable, these cells were cultivated in EB-free medium for 10 passages (45 days). Then the cell culture was reexposed to medium containing 100 μg/ml EB and the growth rates determined. There was no difference between the growth rates of these cells in normal or EB-containing medium. This experiment shows that the EB resistance is maintained during propagation and thus seems to be genetically determined.

One theoretically possible mechanism of resistance could exist in a decreased permeability of the dye into the mutants. The absence of a complete permeability barrier for the cytoplasmic and nuclear compartment of the resistant cells was demonstrated by fluorescence microscopy which localizes EB within the cell. We found intense fluorescence in the nuclei and nucleoli of both the wild-type and resistant cells.

To exclude the possibility that the resistant cells produce a factor capable of inactivating the EB in the medium, we incubated a culture of resistant cells with EB-containing medium for 24 h. The medium was harvested, filtered and supplemented with fresh calf serum and then used for cultivation of wild-type cells. These sensitive cells died within 48 h after exposure to the dye-containing ‘conditioned’ medium. Therefore no destruction of EB in the culture medium by 100 K cells seems to be the basic mechanism of resistance to the dye.

Tumorigenicity

To study the oncogenicity of our drug-resistant SV40-transformed cells 39 Syrian gold hamsters were inoculated with 10⁶ to 10⁷ cells per animal. During an observation period up to 8 months only one hamster developed a specific tumour at the site of inoculation. In comparison 10³ to 10⁴ F5-1 wild-type cells produce a tumour within 3 months following inoculation of Syrian gold hamsters. These results demonstrate that the tumorigenicity of the 100 K cells in the appropriate host system is considerably reduced.

Electron microscopy

Morphological studies of the EB-resistant cells revealed a normal ultrastructure. Only the mitochondrial profiles showed a highly condensed cristae conformation as described by Klietmann et al. (1973) for a cell line resistant to 16 μg/ml EB which also is a derivative of F5-1 cells.
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Mitochondrial DNA

EB exerts in all wild-type cell mitochondria studied a strong inhibitory effect on the synthesis of mitochondrial DNA. In F5-1 cells treated with 16 µg/ml EB no [Me-3H]thymidine was incorporated into closed circular mitochondrial DNA of region I, and this fraction contained almost no circular DNA molecules as detected by electron microscopy (Klietmann et al. 1973b). In contrast, cells resistant to 100 µg/ml EB incorporated [Me-3H]thymidine into circular mitochondrial DNA to a normal amount, which banded in the appropriate density region of the isopycnic CsCl-EB gradient. Closed circular double-stranded molecules of monomeric contour length could be visualized by electron microscopy in the fractions of component I mitochondrial DNA which also contained a small percentage of minicircles (Klietmann et al. 1977; Smith & Vinograd, 1972).

The incorporation of [Me-3H]thymidine into DNA II, which consists of nuclear or nicked and linear mitochondrial DNA was identical in wild-type cells and in mutants (Fig. 3).

Synthesis of mitochondrial DNA in vitro

As shown in Fig. 4A in mitochondria of wild-type cells incorporation of ³H-dTTP into DNA was strongly inhibited by 16 µg/ml EB. In contrast mitochondria of cells resistant to 16 µg/ml EB were found to show no significant inhibition of DNA synthesis in the presence of 16 µg/ml EB after 60 min incubation (Fig. 4B).

Gel electrophoresis

The proteins of the mitochondrial fraction of purified mitochondria of wild-type cells and 100K cells were compared by electrophoresis on SDS-urea polyacrylamide gels. The profiles of polypeptides of the mitochondrial fraction of wild type F5-1 cells showed consistent, characteristic electrophoretic patterns. However when mitochondrial protein of EB-resistant cells were coelectrophoresed and compared, significant differences were observed. Polypeptide 14 (30,000 Daltons) in F5-1 mitochondria profile was absent or greatly reduced in the 100K mitochondria profile (Fig. 5). In addition to the absence of one polypeptide in the 100K mitochondria profile, qualitative variations in the electrophoretic profiles were observed. The relative proportions of polypeptides 12 and 7:8:9 were different in wild-type mitochondria and EB-resistant mitochondria patterns.

Fig. 2. A. Electron micrograph of F5-1 wild-type cells. Mitochondrial profiles appear normal. x 40,000. B. Cloned cells resistant to 100 µg/ml EB, grown in medium containing 100 µg/ml EB. Mitochondria have an essentially normal ultrastructure. x 40,000. C. Electron micrograph of cells resistant to 200 µg/ml EB. The mitochondrial profiles show a highly condensed configuration, cristae are relatively sparse. x 40,000.
Fig. 3. A. Mitochondrial DNA profiles in an isopycnic CsCl-EB gradient. Wild-type cells, grown in normal medium. B. Mitochondrial DNA profiles in an isopycnic CsCl-EB gradient. Cells resistant to 100 μg/ml EB, grown in medium containing 100 μg/ml EB.
Fig. 4A. Incorporation of $^3$H-dTTP into mitochondrial DNA of F5-1 cells: in normal medium, ---; and in medium with 16 μg/ml EB, ---. B. Incorporation of $^3$H-dTTP into mitochondrial DNA of cells resistant to 16 μg/ml EB; in normal medium, ---; and in medium with 16 μg/ml EB, ---.
DISCUSSION

Our results demonstrate that SV40-transformed hamster cells can be adapted to EB concentrations up to 250 µg/ml, a condition which is highly cytotoxic for mammalian cells. The EB resistance is genetically stable. It does not influence the mitochondrial DNA content of the 100K cells nor their morphology. But the ultrastructure of the mitochondria was changed to the condensed or unorthodox conformation. The observed drug resistance to EB seems to result from genotypic changes of the cells, which are maintained during long-term cultivation and which persists during further passaging in the absence of the dye. Such drug-resistant cell clones might emerge by mutation and selection, the drug playing a purely selective role. A fact of special interest is the observation that the drug resistance is combined with a considerable reduction of tumorigenicity of 100K cells in the appropriate host system.

We can consider the following changes of the cell metabolism or constituents that can be made responsible for the expression of genotypic drug resistance.
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Alteration of the cell membranes or in an EB transport system are both molecular mechanisms capable of preventing the penetration of a drug into the interior of a cell. A decreased permeability to an inhibitor has been demonstrated in many mutants resistant to drugs (Bech-Hansen, Till & Ling, 1976). The existence of a complete permeability barrier could not be shown by fluorescence microscopy.

An enzymic alteration of EB could also lead to inactivation of the drug. To exclude this mechanism we conducted experiments with ‘conditioned’ medium from resistant 100K cells, which obviously did not release a factor into the nutrient medium capable of destroying the drug action. The possibility of a non-releasable inactivated EB was not tested.

A permeability barrier in the mitochondrial membranes as a result of a mutation in either the nuclear or mitochondrial genome could explain the results obtained. This concept, that the mitochondrial membranes are altered to impermeability for EB can also explain the EB resistance. To confirm this assumption we plan to isolate the inner and outer mitochondrial membranes and to compare their compositions by gel electrophoresis.

The formation of altered mitochondrial enzymes could be a possible mechanism for the ‘neutralization’ of the toxic drug action. An alteration of the specificity of enzymes – such as DNA polymerase – or an alteration of the affinity for the dye of EB-sensitive enzymes could explain EB resistance. The mitochondrial DNA polymerase is a membrane-associated enzyme, which in mammalian cells is much more sensitive to DNA-intercalating dyes such as EB and acriflavin than the nuclear polymerases (Meyer & Simpson, 1969). A mutation in this enzyme could raise its resistance against the attack of this dye. To test this concept experiments of mitochondrial DNA synthesis in isolated organelles were conducted and showed an EB-insensitive DNA synthesis in cells resistant to 16 μg/ml EB in contrast to EB-sensitivity in mitochondria of wild-type cells. Further studies, however, are needed for a better evaluation of the EB effect on the mitochondrial DNA synthesis in an organelle-free membrane-template-complex using the endogenous DNA polymerase of a mitochondrial lysate. Preliminary experiments suggest that this system represents a possible approach for the solution of this problem.

Similar hypotheses have been considered to explain the molecular mechanisms of EB resistance of yeast and Tetrahymena. The numerous inhibitory properties of EB in biological systems are insufficiently explained by the intercalative model. Mahler & Bastos (1974) and Hixon, White & Yielding (1975) described in addition to intercalation with nucleic acids a metabolic product, a photoprodut or a derivative of EB, which covalently binds to mitochondrial DNA in yeast.

In addition there is evidence for high-affinity binding to membranes (Gitler, Rubalca & Caswell, 1969) enhanced by energy conservation in mitochondria (Azzi & Santato, 1971). Grimwood & Wagner (1976) observed the inhibition of oxidative phosphorylation in mouse liver mitochondria.

The mechanism of EB resistance in mammalian cells, specifically the genetic location on nuclear or cytoplasmic DNA, has not been determined at the present time and further studies must be conducted.
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REFERENCES


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