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SELECTION OF MAMMALIAN CELLS RESISTANT TO A CHLORAMPHENICOL ANALOG

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The study of mitochondrial biogenesis has been greatly advanced by the isolation and characterization of various mutants of mitochondrial function. Genetic analysis of such mutants led to the demonstration of non-Mendelian inheritance and of recombination of cytoplasmic genetic characteristics which are considered to reside on mitochondrial DNA (mtDNA) (for reviews see 1–5). Most of this work has utilized *Saccharomyces cerevisiae*, although *Paramecium* (6) and *Neurospora* (7) have been used in similar studies. It has been only recently, however, that mutants of mitochondrial function in mammalian cells have been isolated (8–10).

The selection of chloramphenicol-resistant mammalian cells by Eisenstadt and co-workers (8–10) was done in the presence of ethidium bromide and chloramphenicol. This technique allowed for the selection of cells which were resistant to chloramphenicol at the level of isolated mitochondrial protein synthesis (8) and whose resistance was a cytoplasmic characteristic (10). Mammalian cells resistant to ethidium bromide have also been reported (11), but the nature of this resistance is not known. Studies of mutants of mitochondrial function are necessary for further elucidation of the nature and function of the mitochondrial genetic system in mammalian cells. This kind of genetic approach needs a readily available method for the selection of mammalian cells with altered mitochondrial function.

The thymidine kinase-deficient mouse cell line LMTK$^-$ (12) has been shown to contain a thymidine kinase activity associated with the mitochondria (13–15). This enzyme is presumably responsible for the incorporation of [^{3}H]thymidine or 5-bromodeoxyuridine (BUdR) (13) specifically into the mtDNA of these cells. The possibility exists that specific incorporation of BUdR into mtDNA may result in mutations of the mitochondrial genome. BUdR has been shown to be an effective mutagen in bacteriophage (16) as well as in animal cell viruses (17, 18). This paper describes the selection and preliminary characterization of LMTK$^-$ cells resistant to Tevenel (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.), the sulfamoyl analog of chloramphenicol (19, 20). Tevenel, a specific inhibitor of mitochondrial protein synthesis, has been shown previously in this laboratory (21–24) to have lesser side effects than D-chloramphenicol, resulting in a more specific effect when applied to the growth of mammalian cells in culture (20).

METHODS

Growth and Maintenance of Cell Cultures

The LMTK$^-$ cell line, originally derived by Kit et al. (12), was obtained from Dr. B. L. Hillcoat of the Department of Biochemistry, McMaster University. The cells were maintained in suspension culture in a minimum essential medium (Joklik's modification) supplemented with 5% fetal calf serum as previously described (20). Cells resistant to Tevenel were also main-

tained in this way; however, the medium usually contained 100 μg Tevenel/ml as well (see below).

Selection of Cells Resistant to Tevenel

Initial experiments were designed to use BUdR as a specific mutagen of mtDNA in the LMTK⁻ cell line. In the course of these experiments it was found that a significant proportion of the LMTK⁻ cell population could be selected for resistance to Tevenel without prior BUdR treatment. BUdR was found not to increase the yield of resistant cells in the uncloned LMTK⁻ cell line. The possible reasons for this lack of effect will be discussed below. This paper deals with cells resistant to Tevenel selected without previous treatment with BUdR.

LMTK⁻ cells growing in suspension culture were treated for 5 days with 100 μg Tevenel/ml. At the end of the 5-day treatment the cells had essentially stopped growing (cf. Fig. 1). The cells were then plated onto 100-mm Petri dishes and maintained in a minimum essential medium (Eagle's) supplemented with 10% fetal calf serum and 100 μg Tevenel/ml. The medium was changed frequently since it became very acidic as cells died off. As a control, mouse L cells were treated in an identical manner. After 2 wk of growth in Tevenel, the LMTK⁻ cell line yielded approximately 80 resistant clones from the 5×10^5 cells plated, while the L-cell line yielded none. The Petri dishes containing the resistant LMTK⁻ cells were allowed to grow to confluence, harvested, and clones selected as described by Puck et al. (25). Three of these clones (denoted T2, T8, and T22) were chosen for analysis.

Measurement of Cell Growth

Cell growth in the presence and absence of 100 μg Tevenel/ml was measured in suspension cultures of LMTK⁻ or Tevenel-resistant cells as described previously (26).

To determine whether the cells resistant to Tevenel retained their thymidine kinase deficiency, the ability of the cells to grow in (*a*) a minimum essential medium (Eagle's) supplemented with 10% fetal calf serum and 25 μg BUdR per ml (12), or (*b*) HAT medium (10^{-4} M hypoxanthine, 5×10^{-5} M thymidine, and 10^{-6} M methotrexate) (27) was examined. Cells deficient in thymidine kinase are able to grow in 25 μg BUdR per ml (12) but not in HAT medium (27).

Measurement of Relative Plating Efficiency

Plating efficiency of LMTK⁻ or resistant cells was determined by scoring the number of clones formed on plates inoculated with 200 cells. The absolute plating efficiency was variable but usually in the range of 70–90%. The relative plating efficiency is reported in order to make the results comparable from experiment to experiment and from cell type to cell type.

Measurement of Protein Synthesis by Isolated Mitochondria

Mitochondria were isolated from 2×10^8 cells essentially as described by Constantino and Attardi (28), except that the cells were washed and disrupted in 0.3 M sucrose, 2 mM EDTA, 2 mM Tris-HCl, pH 7.4, with an Ultra-Turrax homogenizer as described previously (29). Mitochondrial protein synthesis was measured by incubating the mitochondrial fraction at 30°C in a medium (30) which contained 1–2 mg protein/ml, 50 mM KCl, 20 mM potassium phosphate, 5 mM $MgCl_2$, 100 mM sucrose, 10 mM sodium succinate, 2 mM ADP, 0.1 mM in all the amino acids except leucine, 300 μg cycloheximide/ml (28), and 17 μCi L-[4,5-^{3}H]leucine (53 Ci/mmol)/ml, pH 7.2. At time intervals from 0 to 60 min, 0.05-ml samples were removed, put onto Whatmann 3MM filter disks, and then into 5% trichloroacetic acid containing 1 mg unlabeled leucine/ml. At the end of the incubations, the disks were boiled for 20 min in the 5% trichloroacetic acid containing leucine and then washed at 0°C, twice with 5% trichloroacetic acid, once with 50:50 ethanol-ether, and once with ether (31). The filter paper disks were then dried and counted with a toluene-based scintillation fluid in a Nuclear-Chicago Mark I scintillation spectrophotometer (Nuclear Chicago Corp., Des Plaines, Ill.). The effect of increasing concentrations of Tevenel or D-chloramphenicol on mitochondrial protein synthesis was determined in a 30-min incubation. In experiments designed to determine the effect of Triton X-100 on mitochondrial protein synthesis, the incubation medium was modified. ADP and succinate were replaced with 10 mM phosphoenolpyruvate, 2 mM ATP, and 25 μg pyruvate kinase per ml, and the incubations were performed in the presence of 0.01% Triton X-100 (8). The incorporation obtained was approximately 100,000 cpm/mg protein/30 min. Protein was determined by the method of Lowry et al. (32).

MATERIALS

Tevenel was a gift from Dr. C. E. Hoffmann, E. I. Du Pont de Nemours & Co., Inc., Stine Laboratories, Newark, Del.; D-chloramphenicol and L-chloramphenicol were gifts from Parke, Davis & Co., Research Division, Detroit, Mich. Cycloheximide, 5-bromo-2′-deoxyuridine, and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Mo. Phosphoenolpyruvate and pyruvate kinase were products of Calbiochem, La Jolla, Calif. ATP was purchased from Boehringer Mannheim Corp., New York, and ADP from P-L Biochemicals, Milwaukee, Wis. All tissue culture supplies were purchased from Grand Island Biological Co., Grand Island, N. Y. L-[4,5-^{3}H]leucine was a product of Amersham/Searle Corp., Arlington Heights, Ill. The source of hypoxanthine, thymidine, and methotrexate (HAT medium) is as described previously (26).

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RESULTS

Growth Characteristics of Resistant Cells

The growth of three Tevenel-resistant cell lines (T2, T8, and T22) were compared with that of LMTK$^-$ in the presence and absence of 100 μg Tevenel/ml. Typical growth curves for LMTK$^-$ (A) and T8 (B) are shown in Fig. 1. While LMTK$^-$ ceases to grow in 100 μg Tevenel/ml, T8 continues to grow at the same rate in the presence or absence of Tevenel. The same result was obtained for both T2 and T22. The resistant cell lines have been maintained for many weeks in the presence of Tevenel without any apparent effect on cell growth.

To determine whether the resistant cells were cross-resistant to D-chloramphenicol, relative plating efficiencies were determined. In Table I, it can be seen that T8 and T22 plate efficiently in 100 μg Tevenel/ml and 50 μg D-chloramphenicol/ml, while no clones are observed for LMTK$^-$ under these conditions. The slightly lower plating efficiency of the resistant cells in D-chloramphenicol may not be due to a specific effect of the chloramphenicol on mitochondrial protein synthesis, since a lower efficiency is also seen in the presence of 50 μg L-chloramphenicol/ml for T8 and T22 as well as LMTK$^-$. L-Chloramphenicol is not effective in inhibiting mitochondrial protein synthesis but it, as well as D-chloramphenicol, inhibits respiration as described previously (21–24).

The stability of the change responsible for the resistance of these cells was examined by comparing the relative plating efficiencies in the presence and absence of 100 μg Tevenel/ml. A culture of T22 grown for more than 100 days in the absence of Tevenel (about 100 generations) plated with similar efficiencies in the presence and absence of Tevenel, as did a culture of T22 maintained in Tevenel for more than 1 mo (Table II). This result suggests that the change responsible for Tevenel

TABLE I

Effect of Chloramphenicol Analogs on the Relative Plating Efficiencies of LMTK$^-$, T8, and T22

Drug	Relative plating efficiency		
	LMTK$^-$	T8	T22
None	1.00 ± 0.04	1.00 ± 0.12	1.00 ± 0.05
Tevenel	0	0.96 ± 0.08	0.87 ± 0.07
D-Chloramphenicol	0	0.74 ± 0.08	0.68 ± 0.08
L-Chloramphenicol	0.71 ± 0.07	0.86 ± 0.13	0.78 ± 0.11

Relative plating efficiency was determined as described in Methods. The concentration of Tevenel is 100 μg/ml, and the concentration of L- and D-chloramphenicol is 50 μg/ml.
The errors indicate standard deviation. The absolute plating efficiencies in the absence of drug were 76%, 78%, and 68% for LMTK$^-$, T8, and T22, respectively.

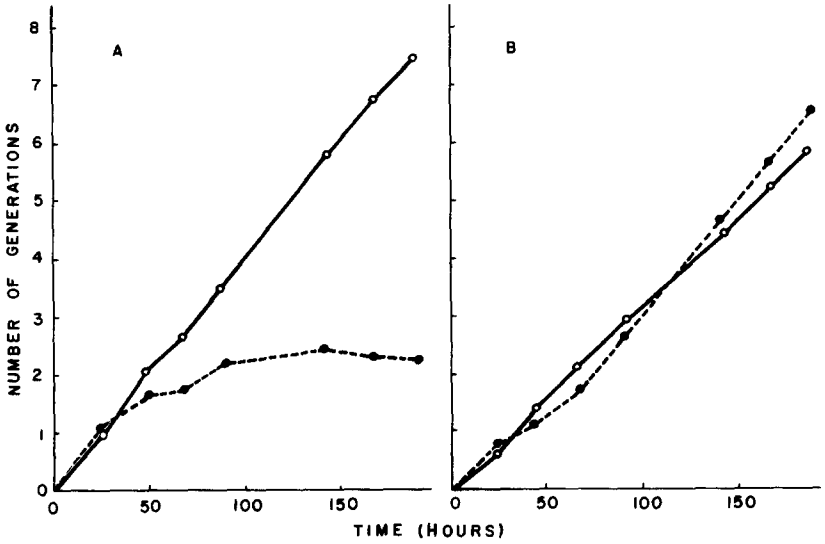

FIGURE 1 Growth curves of LMTK$^-$ cells (A) and T8 cells (B). Growth measurements were performed on cells growing in suspension culture as described previously (19). Each cell count is the average from two cultures. Tevenel (100 μg/ml) was present (●----●) or absent (O——O) during growth.

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TABLE II

Stability of the Resistance to Tevenel in T22

Drug	Relative Plating Efficiency	
	T22 (−)*	T22 (+)‡
None	1.00 ± 0.06	1.00 ± 0.05
Tevenel	0.92 ± 0.04	0.98 ± 0.13

Relative plating efficiency was determined as described in Methods. The concentration of Tevenel is 100 μg/ml. The errors indicate standard deviation. The absolute plating efficiencies in the absence of Tevenel were 89% and 78% for T22 (−) and T22 (+), respectively.

* T22 (−) maintained for 100 days in the absence of Tevenel.

‡ T22 (+) maintained for 35 days in the presence of 100 μg Tevenel/ml.

resistance is stable over the time interval examined and is inheritable. Similar results have been found for the other two resistant lines.

The resistant cell lines were examined for their ability to grow in the presence of BUdR and HAT. They were found to be resistant to 25 μg BUdR/ml and sensitive to HAT, consistent with retention of the thymidine kinase deficiency of the parent cell line (LMTK$^-$).

Mitochondrial Protein Synthesis

To determine that the growth characteristics of the resistant cells is not due to changes in cell membrane permeability to Tevenel and D-chloramphenicol, the effect of these drugs on protein synthesis in isolated mitochondria was examined. As can be seen in Fig. 2, protein

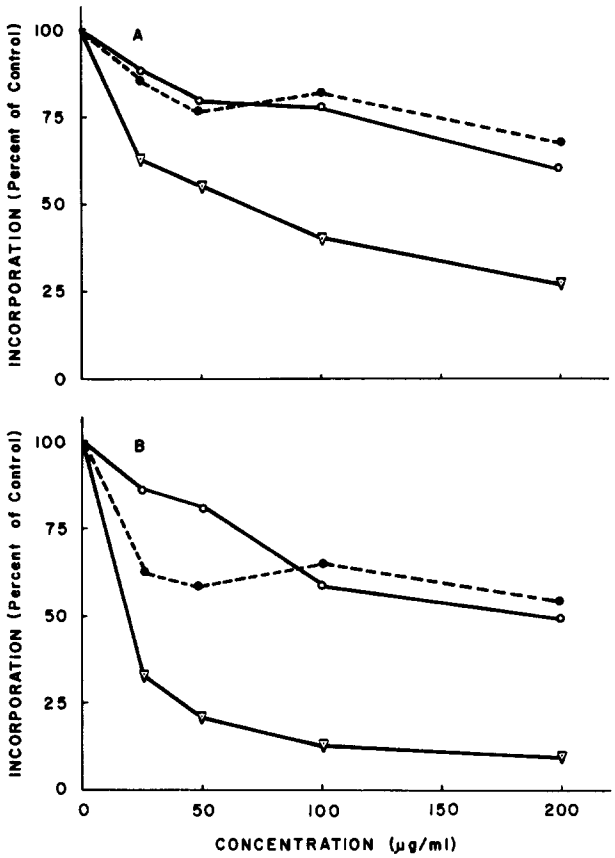

FIGURE 2 Effect of increasing concentrations of Tevenel (A) or D-chloramphenicol (B) on protein synthesis in isolated mitochondria from LMTK$^-$ (▽——▽), T8 (○——○), or T22 (●····●). Protein synthesis was measured as described in Methods; incubations were carried out for 30 min at 30°C; 100% incorporation is approximately 100,000 cpm/mg protein/30 min.

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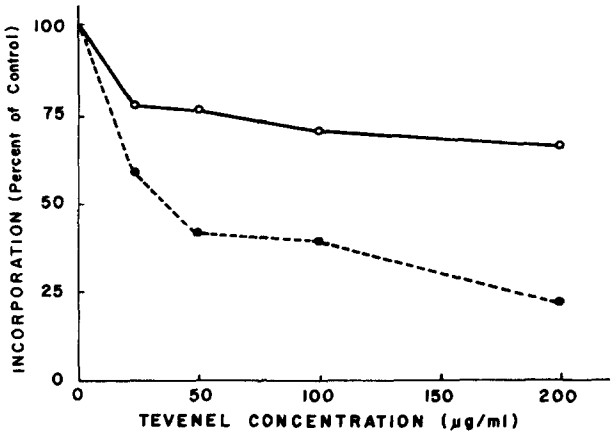

FIGURE 3 Effect of increasing concentrations of Tevenel on Triton X-100- (0.01%) treated mitochondria from LMTK⁻ (●----●) or T22 (O——O). Protein synthesis was measured as described in Methods and Fig. 2.

synthesis by mitochondria from both T8 and T22 shows a marked resistance to both Tevenel and D-chloramphenicol with respect to the protein synthesis of LMTK⁻ mitochondria. Although the resistance is not complete, similar results have been reported for chloramphenicol-resistant HeLa cells (8), and for chloramphenicol-resistant yeast (33). The resistance to Tevenel of the mitochondrial protein synthesis in T22 was not decreased by incubation in the presence of 0.01% Triton X-100 (Fig. 3), suggesting that the resistance is not due to a change in the permeability of the mitochondrial membranes to the drug (8). Similar results were obtained with T8 mitochondria.

DISCUSSION

The Tevenel-resistant cells described above have many of the properties expected for cells carrying a cytoplasmic mutation. The resistance is stable; it is expressed at the level of isolated mitochondrial protein synthesis; it does not appear to be due to a lack of permeability of the mitochondrial membrane to Tevenel, and both cell growth and mitochondrial protein synthesis are cross-resistant to D-chloramphenicol. The ultimate proof that the resistance is a mutation within the mtDNA would be to demonstrate cytoplasmic inheritance of the characteristic, as has been shown for resistant mouse A9 cells by Bunn et al. (10). Experiments along this line are now in progress.

The ease with which Tevenel-resistant cells can be isolated from an LMTK⁻ population may make this cell line useful for the isolation of cells resistant to other drugs which interfere with mitochondrial function. Isolation of such mutants would then allow studies on recombination of the mammalian mitochondrial genome. Evidence that recombination does occur in mammalian mtDNA has been presented by Coon et al. (34), and Dawid et al. (35) studying the buoyant density of mtDNA in rodent-human hybrids. Hybrid cells may offer a convenient system for the study of recombination of antibiotic-resistant markers in mammalian mtDNA.

The reason that BUdR was not found to stimulate the production of cells resistant to Tevenel is not clearly understood. It is possible that the population of LMTK⁻ cells chosen for study contained a very high proportion of resistant cells due to its prior exposure to BUdR and that this genetic lesion was maintained throughout many generations in the absence of the selective pressure. A high background of resistant cells would then mask any effect of BUdR in producing new mutations. It is also not known what the effect of ethidium bromide was in the selection of chloramphenicol-resistant cells, as reported by Spolsky and Eisenstadt (8).

The selection conditions reported here are significantly different from those reported by Spolsky and Eisenstadt (8). Here, the LMTK⁻ cells were exposed to inhibitor for 5 days before plating in Tevenel. This treatment was done in order to inhibit the growth of sensitive cells before plating, such that they did not overgrow the Petri dishes. This procedure could tend to enrich the suspension

culture with resistant cells, and would account, in part, for the high frequency of clones observed under these conditions. In addition, the use of Tevenel rather than chloramphenicol is significant. It has been shown previously (20) that chloramphenicol is more inhibitory than Tevenel on the growth of cells in culture. This effect is probably a result of the inhibition of respiration (21–24), or perhaps of DNA synthesis (36) by chloramphenicol. It may be more difficult, therefore, to select for cells resistant to chloramphenicol than to Tevenel. These differences in selection conditions may account for the failure of Bunn et al. (10) to observe chloramphenicol-resistant LMTK$^-$ cells.

SUMMARY

This study describes the selection and preliminary characterization of mammalian cells resistant to 100 μg Tevenel/ml. Tevenel, the sulfamoyl analog of chloramphenicol, is a specific inhibitor of mitochondrial protein synthesis.

After growth in suspension culture for 5 days in 100 μg Tevenel/ml and subsequent plating in 100 μg Tevenel/ml, LMTK$^-$ cells yielded resistant clones. As a control, L cells treated identically yielded no clones. Three resistant clones were chosen for study. Each resistant cell line had an identical growth rate in the presence and absence of 100 μg Tevenel/ml. By plating efficiency analysis, the resistant cells were found to be cross-resistant to D-chloramphenicol. The change responsible for resistance was found to be stable for at least 100 generations in the absence of the drug.

Protein synthesis by isolated mitochondria of resistant cells was found to be less inhibited by concentrations of both Tevenel and D-chloramphenicol up to 200 μg/ml than the protein synthesis by LMTK$^-$ mitochondria. This resistance in vitro was not changed by incubation of the mitochondria in 0.01% Triton X-100.

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