TRYPTOSE PHOSPHATE BROTH CONFERS TO CHICK EMBRYO CELLS RESISTANCE TO THE INHIBITORY EFFECT OF CHLORAMPHENICOL ON GROWTH

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Summary: Tryptose phosphate broth (10% V/V) prevents the inhibitory effect of chloramphenicol (80 ug/ml) on the growth of chick embryo fibroblasts. Cellular cytochrome c oxidase activity is rapidly lost indicating that chloramphenicol inhibits the mitochondrial protein-synthesizing system in the presence of the broth. To compensate for the apparent decreased mitochondrial ATP production, pyruvate kinase and lactate dehydrogenase activities, 2-deoxyglucose uptake and lactic acid produced in treated cells are greater than those in the control cells during the first few generations. These fermentation values tend to revert to normal during later cell generations.

It is now well established that, in eukaryotic cells, the antibacterial antibiotic chloramphenicol blocks specifically the mitochondrial protein-synthesizing system without altering that of the cytoplasm (1-3). This drug, and specific inhibitors of the cytoplasmic protein-synthesizing system such as emetine and cycloheximide, have been extensively used to study the biogenesis of mitochondrial proteins in Saccharomyces cerevisae and other facultative anaerobic yeasts as well as their cytoplasmic mutant lacking a functional mitochondrial protein-synthesizing system (4-7).

Chloramphenicol has also been used to study the biogenesis of mitochondrial proteins in animal cells. Experiments of this sort, however, have been found to be of limited value since these obligate aerobic cells reach a stationary phase after 3 to 4 cell generations in chloramphenicol (3,8-10) and often degenerate after prolonged exposure to the drug. It is generally assumed that the cessation of animal cell growth in the presence of inhibitors of the mitochondrial protein-synthesizing system occurs when the preexisting respiratory capacity of the cell, usually assayed by cytochrome c oxidase activity, is diluted to the point that it becomes rate-limiting. The present report indicates that chick embryo fibroblasts treated with chloramphenicol

might, under proper conditions, behave phenotypically as facultative anaerobic cells and grow in culture with mitochondria depleted of cytochrome c oxidase activity.

Materials and methods

Chick embryo fibroblasts were prepared from 9 to 12-day-old White Leghorn embryos and cultivated as previously described (11). Secondary cultures of chick embryo fibroblasts were trypsinized soon after confluence, collected by centrifugation and resuspended in medium 199 (12) or MEM (13) supplemented with inactivated fetal calf serum (6%) or in Ham's F12 (14) medium supplemented with 10% tryptose phosphate broth (Difco), 8% calf serum and 2% inactivated chick serum. All medium also contained 100 IU/ml of penicillin and 100 ug/ml of streptomycin. The cells were seeded at 1×10^5 cells/ml into Falcon plastic flasks (75 cm^2) and incubated at 39°C in a CO_2 incubator. The next morning, cells attached to the surface of a few flasks were trypsinized and counted. The medium of the other flasks was replaced by fresh medium containing no addition or chloramphenicol (80 ug/ml). Cultures were refed daily with fresh medium to ensure adequate nutrition. Proliferation of chick embryo fibroblasts was determined by counting the cells present in the medium every day and those detached from the confluent monolayer by trypsin.

To determine the effect of the inhibitors on cytochrome c oxidase, attached cells were removed with trypsin, counted and centrifuged. The cells were washed twice with 0.25 M sucrose, counted, suspended in distilled water and transferred to a Dounce homogenizer (11). The homogenate obtained was used to determine the activity of cytochrome c oxidase (11). Pyruvate kinase and lactate dehydrogenase activity was determined as described (15,16) on cells detached from the flask surface by scraping. The amount of ^{3}H-2-deoxyglucose taken up by the chick cells was determined according to the method of Kletzien and Perdue (17). Lactic acid released in the incubation medium was assayed enzymatically by the use of reagent kits of Sigma Chem. Co. Protein concentrations were determined by the Lowry method (18) using bovine serum albumin as a standard.

Results

Chick embryo cells cultured in medium 199 or MEM grew for only 2 to 4 generations in the presence of the inhibitor chloramphenicol (Fig. 1, a, b, (11)). These results were as expected, a similar number of cell generations being observed for animal cells from quite different sources and species treated with the same drug (3,17). When chick embryo fibroblasts were cultivated in medium F12, however, they were found to respond to chloramphenicol quite differently. As shown in Fig. 1c, there was no cessation of cell growth in the presence of chloramphenicol, although the growth rate of the treated chick cells was obviously less than that of the control, with a generation time of 30 hrs compared to 20.

The long-term growth of chloramphenicol-treated chick fibroblasts was rendered possible by the presence in the incubation medium of the tryptose phosphate broth.

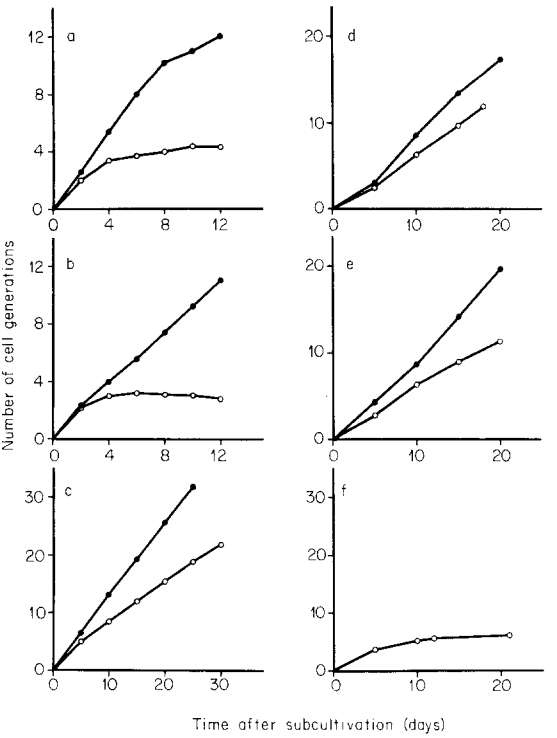

Fig. 1- Growth of chick embryo fibroblasts in the presence of 80 ug/ml of chloramphenicol. (a) and (d), medium 199; (b) and (e), medium MEM; (c) and (f), medium F12. Tryptose phosphate broth (10% V/V) was present in (c), (d) and (e). (●), control; (o), chloramphenicol.

As seen in Fig. 1, d, e, its addition to medium 199 and MEM prevented the inhibitory effect of chloramphenicol, the cells growing here also at a rate slightly inferior to that of the untreated fibroblasts. In the absence of the broth, cells in medium F12 failed to grow more than 4 generations (Fig. 1, f) and thus behaved as those cultivated in unsupplemented medium 199 and MEM (Fig. 1, a, b).

That tryptose phosphate broth did not exercise its action by preventing the inhibitory effect of chloramphenicol on the mitochondrial protein synthesizing system is shown by the behaviour of cytochrome c oxidase activity (Fig. 2). It would be expected that if the synthesis of the subunits of this enzyme coded for by mitochondria DNA (3) was inhibited by chloramphenicol, then its activity per cell, and its specific activity as well would decrease to 50% in one generation, to 25% in two generations,

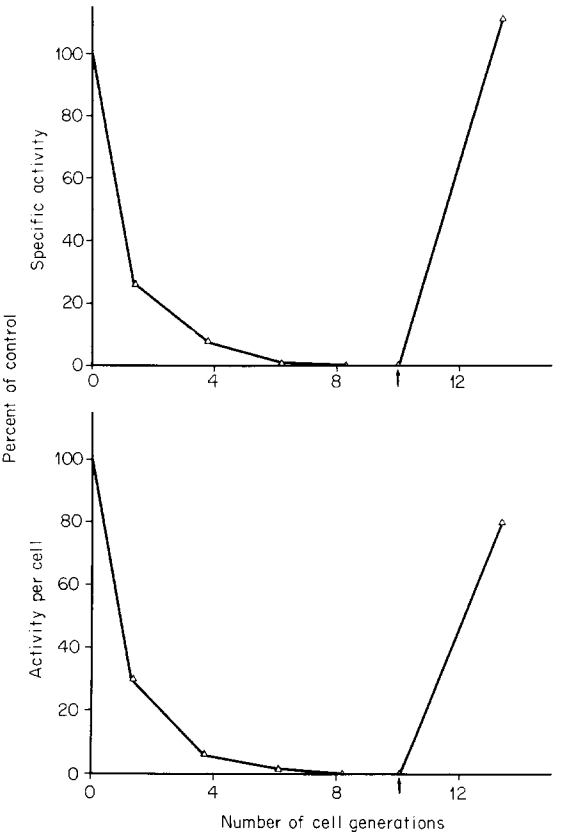

Fig. 2- Change in activity per cell and specific activity of cytochrome c oxidase during long-term growth of chick embryo fibroblasts in medium F12 containing tryptose phosphate broth and chloramphenicol. At the point indicated by the arrow, the monolayer was washed twice and resuspended in fresh medium without chloramphenicol. The mean absolute value for 10^7 control cells is 6.1 nmoles cytochrome c oxidized/min.

and so on. As shown in Fig. 2, the decrease in cytochrome c oxidase specific activity or activity per cell was as expected, becoming undetectable by the 8th generation, and thus indicating that under the present experimental conditions, mitochondrial translation was indeed inhibited. Upon removal of chloramphenicol, cytochrome c oxidase activity resumed suggesting that no apparent lesions have been introduced in the mitochondrial protein-synthesizing system. Similar results were obtained when the chick cells were cultivated in medium 199 or MEM.

Data presented in Fig. 3 show that chloramphenicol-treated chick cells had

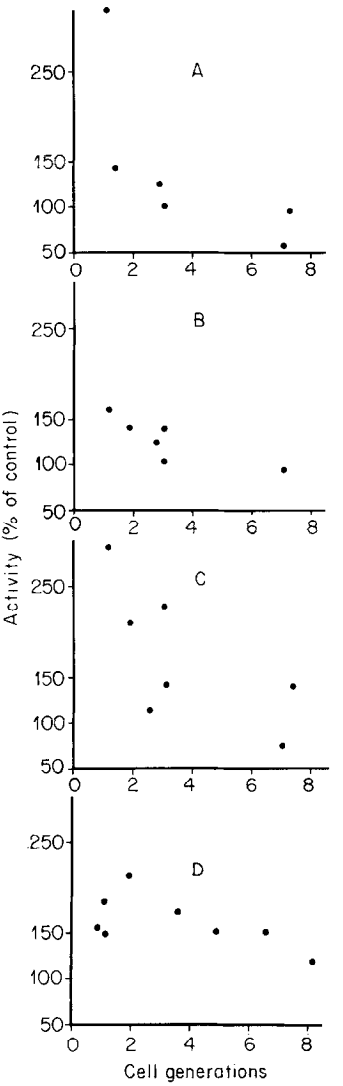

Fig. 3- Change in activity per cell of pyruvate kinase (A), lactate dehydrogenase (B) ^{3}H-2-deoxyglucose uptake (C) and lactic acid produced (D) during long-term growth of chick embryo fibroblasts in medium F12 containing tryptose phosphate broth and chloramphenicol. The mean absolute value for 10^{7} control cells are 0.6 and 2.5 umoles of NADH oxidized/min for pyruvate kinase and lactate dehydrogenase respectively, 0.04 nmole of ^{3}H-2-deoxyglucose uptake/30 min and 2.7 umole of lactic acid produced/30 min. The mean cell number per flask (75 cm^{2}) was 5.5 x 10^{5} for A, B and C and 2.5 x 10^{6} for D. The values shown derived from two (A, B, C) and four (D) different experiments.

increased pyruvate kinase and lactate dehydrogenase activities and take up 2-deoxyglucose and release lactic acid at a higher rate than the control cells. This was particularly evident in the first few generations of growth, where the mitotic rate

of treated and untreated cells tended to be similar. As the growth rate of the treated cells decreased, so did the glycolytic enzyme activities, 2-deoxyglucose uptake and lactic acid release.

Discussion

The present results indicate that tryptose phosphate broth confers to chick embryo fibroblasts resistance to the growth inhibitory effect of chloramphenicol. Preliminary results from this laboratory indicate that quail and chinese hamster embryo fibroblasts treated with chloramphenicol behave in the same way as do chick embryo fibroblasts when tryptose phosphate broth is added to the culture medium. These observations indicate that the protective effect of the broth is not specific to the chick cells and can be extended to other animal cells as well. They further raised the possibility of using animal cells as biological systems to study the biogenesis of mitochondrial proteins.

The mechanism(s) through which tryptose phosphate broth exercises its action is unknown. The broth does not prevent the inhibitory effect of chloramphenicol on the mitochondrial protein-synthesizing system as demonstrated by the reduction in cytochrome c oxidase activity to an undetectable level in the treated cells. Decreased capacity for mitochondrial ATP production which should result under these conditions, seems to be compensated for by increased fermentation, at least during the first few cell generations, since a rapid increase of pyruvate kinase and lactate dehydrogenase activities, 2-deoxyglucose uptake and lactic acid release is observed. It remains to be seen whether the increased glycolysis is mediated through the tryptose phosphate broth or is a direct consequence of the inhibition of the mitochondrial protein-synthesizing system by chloramphenicol. The observation that fermentation values tend to revert to normal as chloramphenicol-treated cells continue to divide may indicate that the ATP produced through this pathway is apparently sufficient to cope with the slower growth of these cells. However, the possibility that a certain amount of ATP could be generated through a respiratory pathway lacking cytochrome aa_3 cannot be eliminated. Evidence of such a pathway **has** been found in plants and microorganisms treated with inhibitors of the mitochondrial protein-synthesizing system (20-26).

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