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CHICK EMBRYO CELLS RENDERED RESPIRATION-DEFICIENT BY CHLORAMPHENICOL AND ETHIDIUM BROMIDE ARE AUXOTROPHIC FOR PYRIMIDINES

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Summary: The present results demonstrate that uridine confers on cultured chick embryo cells resistance to the growth inhibitory effect of chloramphenicol and ethidium bromide. Cellular cytochrome oxidase activity is lost suggesting that uridine does not prevent the inhibitory effect of the drugs on mitochondrial transcription and translation. Other than cytidine, none of the precursors and derivatives of uridine tested supports cell growth.

We have recently demonstrated (1-3) that populations of chick embryo fibroblasts (CEF) cultivated in the presence of tryptose phosphate broth (TPB) are inherently resistant to the growth inhibitory effect of chloramphenicol (CAM) and ethidium bromide (EB). In the absence of the broth, CEF behave essentially the same as other animal cell populations treated with these drugs (4-7) and their growth, after a few generations, either comes to a halt or is severely reduced. Biochemical analyses and electron microscopic observations revealed that TPB did not prevent the inhibitory effect of the drugs on mitochondrial macromolecular-synthesizing systems (1-3). The cells grow with mitochondria devoid of a functional respiratory chain.

For some time, we have been engaged in identifying the active component(s) present in TPB, an enzymatic digest of animal extracts supplemented with dextrose, sodium chloride and disodium phosphate (Difco Manuel, '53). We report here that the active components of the broth are of pyrimidine origin. A detailed account of the various physico-chemical methods used to elucidate the nature of the components and their structure will be reported elsewhere. In the present paper, we demonstrate that uridine and cytidine confer on chick embryo cells resistance to the growth inhibitory effect of CAM and EB.

Abbreviations: CEF, chick embryo fibroblasts; TPB, tryptose phosphate broth; CAM, chloramphenicol; EB, ethidium bromide.

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Materials and methods

Chick embryo cells were prepared from 8- to 11-day-old White Leghorn embryos as described previously (2). Cells were cultivated in Ham's F12 medium supplemented with 8% and 2%, respectively, calf and inactivated chicken sera (medium 8-2). Penicillin (100 I.U./ml) and streptomycin (100 µg/ml) were routinely added to the culture medium. Growth curves, cell attachment and colony formation assays, protein determination and cytochrome oxidase activity measurements were done as described (1-2). Growth medium, sera, penicillin, streptomycin and trypsin were purchased from Gibco, Grand Island, N.Y. Uridine and cytidine were obtained from BDH Chem. Poole, England and dihydroorotic acid and orotidine from Calbiochem, La Jolla, Calif. All other precursors and derivatives of uridine were from Sigma Chem. Co., Saint Louis.

Results

Figure 1 shows typical proliferative curves of populations of chick embryo cells cultivated with (medium 8+2+Urd) or without (medium 8+2) uridine. In the experiment shown, the mean doubling time of the culture in both media was approximately 20 hours. When CAM (100 µg/ml) was added to medium 8+2 (Fig.1a), the cell population doubling time for the first three generations was similar to control but rose thereafter to over 250 hours. Chick embryo cell populations treated with EB (0.2 µg/ml) behave the same as those treated with CAM. The population doubling time for the

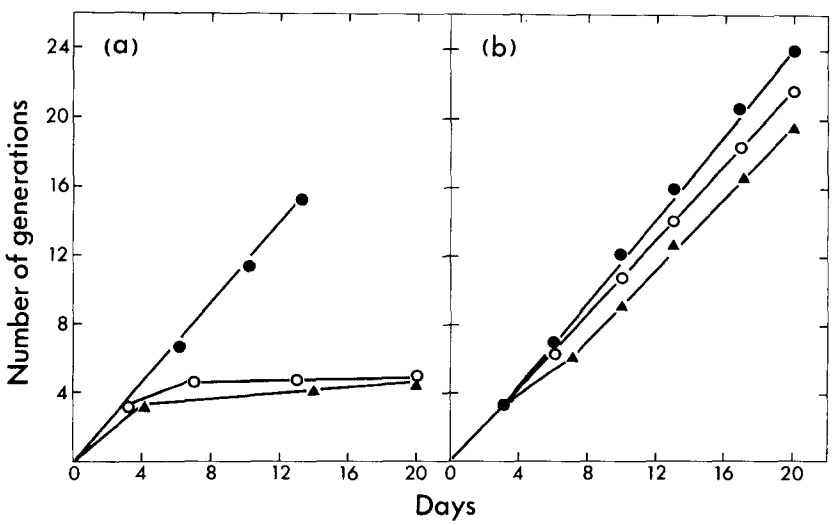

Figure 1: Effect of chloramphenicol and ethidium bromide on long-term growth of chick embryo cell populations cultivated (a) in the absence of uridine (medium 8-2) and (b) in the presence of uridine (medium 8-2-Urd). Cells were grown by successive transfers at inoculation densities of 1.0 to 2.0 x 10^6 cells/20 ml into 75 cm^2 plastic flasks. ●—●, control; chloramphenicol (100 µg/ml), o——o; ethidium bromide (0.2 µg/ml), ▲—▲. Growth curves were determined as described(2).

first 3 to 4 generations was slightly increased as compared to control and rose thereafter to over 175 hours (Fig.1a).

The addition of uridine (2.0 μg/ml) into the incubation medium prevents the growth inhibitory effect of CAM and EB (Fig.1b). The mean population doubling time was slightly increased to 22 hours in the presence of CAM and to 25 hours in the presence of EB. Cell attachment efficiency, that is the number of seeded cells which were found to be attached to the plastic surface five hours after seeding (2), was approximately the same in all media (Table 1). As compared to control, however, the mean cloning efficiency values of CEF populations treated with the drugs were found to be reduced by approximately 25% (Table 1). The colony size was generally slightly smaller than that in media devoid of the drugs.

We have previously demonstrated that cytochrome oxidase activity of CAM- and EB-treated CEF populations cultivated in the presence of TPB became undetectable after 6 to 8 cell doublings (1-3). These and other observations (2,3) have been interpreted to mean that the broth does not interfere with the well known inhibitory effect of the drugs on mitochondrial transcription and translation (8-11). As reviewed by Schatz and Mason (12), some of the subunits of the enzyme are coded for by mitochondrial DNA and elaborated on mitoribosomes. That uridine also does not interfere with the inhibitory effect of CAM and EB on mitochondrial macromolecular-synthesizing systems was evidence by the absence of detectable cytochrome oxidase activity in CEF populations treated with the drugs for 7 to 20 generations (Table 1).

As shown in table 2, the rate of growth of CEF populations treated with CAM is a function of uridine concentration in the medium. Maximal growth was obtained at concentrations of uridine equal to or higher than 2.0 μg/ml. None of the uridine precursors tested was able to support cell growth. Of the four derivatives of uridine tested (thymidine is present in Ham's F12 medium), only cytidine showed activity. Uracil and cytosine were inactive. CEF treated with EB responded in the same way as CAM to the different precursors and derivatives of uridine tested.

TABLE 1

Effect of chloramphenicol and ethidium bromide on cell attachment efficiency, colony formation and cytochrome oxidase activity of chick embryo cells cultivated in the presence of uridine

Cells cultivated in	Cell attachment efficiency (%)	Number of colonies scored	Cytochrome oxidase activity nmoles cyt. c oxidized/min/mg prot.
Medium 8-2+Urd	83 ± 11	366 ± 10	47 ± 4
Medium 8-2+Urd+CAM	84 ± 2	280 ± 28	<0.5
Medium 8-2+Urd+EB	79 ± 3	264 ± 23	<0.5

Cultures in media 8-2+Urd and 8-2+Urd + drug for 7 to 20 generations were used in this study. Cell attachment efficiency is defined as the percentage of the seeded cells which are found attached to the plastic surface five hours after seeding. The number of cells attached at this time was used to determine the number of cell doublings attained by the population at confluence as described (2). The values are the mean ± SDM of 4 different experiments. For colony formation assay, cells were trypsinized, diluted with fresh medium and seeded (2000 cells) in 25 cm^2 plastic flasks. Colonies were scored on day 6 or 7. The values are the mean ± SDM of 4 different experiments done in triplicate. For cytochrome oxidase activity, cultures were trypsinized, centrifuged, washed twice with 0.25M sucrose and resuspended in water. The cell suspension was transferred to a Dounce homogenizer and a few up-and-down strokes of the piston were sufficient to break all the cells. The homogenates obtained were used as source of enzymes. The values reported are the mean ± SDM of three experiments.

TABLE 2

Effect of chloramphenicol on the doubling time of populations of chick embryo cells cultivated in the presence of increasing concentrations of uridine or of various precursors and derivatives of uridine

Compound	Concentration µg/ml	Population doubling time (hours)
Uridine	0.25	65
Uridine	0.5	45
Uridine	1.0	30
Uridine	2	24
Uridine	8	24
Cytidine	2	27
Carbamoyl phosphate	20[x]	250
Carbamoyl-DL-aspartate	20[x]	250
Dihydroorotic acid	20[x]	250
Orotic acid	20[x]	250
Orotidine	20[x]	250
Deoxyuridine	20[x]	250
Deoxycytidine	20[x]	250
Uracil	20[x]	250
Cytosine	20[x]	250

Experimental procedure was as described in the legend of figure 1. Concentrated solutions of the compounds were made up in water and the pH of the solutions adjusted to 7.0. The solutions were kept at 4°C for 2 to 3 weeks before being discarded. The population doubling times were calculated from CEF cultivated in the presence of CAM for more than 4 generations. The values are the mean of 2 or more experiments.

[x] Concentrations ranging from 2 to 20 µg/ml were tested and all gave population doubling times of about 250 hours.

Discussion

The results reported herein clearly demonstrate that uridine confers on chick embryo cells resistance to the inhibitory effect of CAM and EB on growth. Absence of cytochrome oxidase activity in long-term drug-treated CEF populations suggest that uridine does not prevent the inhibitory effect of the drugs on mitochondrial macromolecular-synthesizing systems. The cell populations appear to grow with mitochondria devoid of a functional respiratory chain. No lag or adaptation period

was required for CEF to proliferate in the presence of the inhibitors suggesting that the cell population as a whole was inherently resistant to the drugs and proliferation did not result from the selection of a particular cell type. This was supported by cell attachment and cloning efficiency values of the drug-treated cell populations which were relatively constant from the 7^{th} to the 20^{th} generations. The present observations are thus essentially similar to those we reported recently (1-3) and which demonstrated that CAM- and EB-treated CEF are inherently resistant to the growth inhibitory effect of the drugs in the presence of TPB.

The present results suggest that CEF rendered respiration-deficient by CAM or EB are unable to catalyze de novo synthesis of UMP. This nucleotide is the end product of a biosynthetic pathway which involves 6 different enzymes. At least three possibilities may be raised to explain the chick cell requirement for uridine: (a) the drugs could be inhibitors (competitive or non-competitive) of one or many of the different enzymes involved in the biosynthesis of UMP. There are many examples in the literature where pyrimidine auxotrophy is induced by inhibitors of the activity of these enzymes (13, 14); (b) one or many of the enzymes of the pyrimidine pathway are under some type of regulation by information encoded in mitochondrial DNA. Some observations have led to conclusions that mitochondrial genetic information may play a significant role in the regulation of the activity of nuclear genes (15-17); (c) since mitochondria are multifunctional organelles (18), it may be that disorganization of the mitochondrial structure by CAM and EB affects the activity of enzymes or sequences of enzymes whose end products are required in the biosynthetic pathway of uridine. Interestingly, the enzyme dihydroorotic acid dehydrogenase, which catalyzes the reversible conversion of dihydroorotic acid to orotic acid, has been reported to be located on the outer surface of the inner membrane of mitochondria (19). It is a very complex dehydrogenase whose mechanism of action is still unclear (20-21). There are indications, however, that its activity in vivo is linked to a functional electron transport chain (21).

Preliminary attempts to pinpoint a defective enzymatic step have been made by cultivating CAM-treated CEF in the presence of known precursors of UMP. None of

them support cell growth. These observations suggest either that a general depression of the several enzymes involved in UMP biosynthesis is induced by the drugs or that the last enzyme of the pathway, orotidine-5'-phosphate decarboxylase which catalyzes the conversion of orotidilic acid to UMP, is inoperative. The observations of Skehel et al (22) that orotic acid and uracil are poorly incorporated, if at all, by cultured chick embryo cells have to be taken also into consideration. To our knowledge, it has not yet been reported whether or not CEF are able to incorporate other precursors of uridine. Such incorporation experiments coupled with a measure of the activity of the various enzymes involved in uridine biosynthesis should be able to pinpoint the defective enzymatic step(s) in the drug-treated cells and shed light on the mechanism responsible for pyrimidine auxotrophy.

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