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IRREVERSIBLE INHIBITION OF THE EARLY INCREASE IN ORNITHINE DECARBOXYLASE ACTIVITY FOLLOWING GROWTH STIMULATION IS REQUIRED TO BLOCK EHRLICH ASCITES TUMOR CELL PROLIFERATION IN CULTURE

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SUMMARY

Ehrlich ascites tumor cell growth in culture was inhibited by α-difluoromethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase, provided that treatment was initiated at the time of growth stimulation. When α-difluoromethylornithine was added after the activation of polyamine synthesis caused by the growth stimulus, i.e. when a 3-5-fold increase in putrescine and spermidine content had occurred, cell proliferation was completely unaffected. α-Methylornithine, a competitive inhibitor of ornithine decarboxylase, did not affect cell proliferation even when added at the time of growth stimulation. Compared to α-difluoromethylornithine, α-methylornithine only produced a partial and transient decrease in the cellular putrescine and spermidine content.

INTRODUCTION

The recent development of specific polyamine synthesis inhibitors has provided a basis for the evaluation of the role played by the polyamines putrescine, spermidine and spermine in growth and development (1). Depletion of the cellular putrescine and spermidine content by α-methylornithine or α-difluoromethylornithine, inhibitors of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17), the initial and rate-limiting enzyme in polyamine synthesis, has been shown to markedly inhibit growth and division of mammalian cells in culture (2-8).

The present study was designed to determine the effect of reversible inhibition of ornithine decarboxylase (by means of α-methylornithine treatment) (2, 9) compared to irreversible inhibition (by means of α-difluoromethylornithine treatment) (4, 10) on Ehrlich ascites tumor cell proliferation in culture. We also attempted to reveal whether activation of polyamine synthesis prior to treatment with an inhibitor of ornithine decarboxylase would abolish its antiproliferative effect.

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MATERIALS AND METHODS

A hyperdiploid subline of the Ehrlich ascites tumor was adapted to suspension culture growth in 75 cm^2 Falcon flasks. The cultures were incubated without agitation at 37°C in a water-saturated atmosphere of 95 % air and 5 % CO_2. The growth medium used (Swim's S-77) was supplemented with 11.1 mM D-glucose, 0.1 mM L-cystine, 2 mM L-glutamine, 0.1 mM sodium pyruvate, 1.8 mM $CaCl_2$, 17.5 mM $NaHCO_3$, phenol red (0.001 %), penicillin (50 IU/ml), streptomycin (50 µg/ml), and finally, when the pH had been adjusted to 7.4, fetal calf serum (10 %). The cells were subcultured every 3 or 4 days by a 20-fold dilution with fresh medium (from 2 x 10^6 to 1 x 10^5 cells per ml). During exponential growth the cell number doubled in approximately 13 hr.

In one experiment, 1 x 10^7 plateau phase cells were suspended, at time 0, in 100 ml of fresh growth medium containing either 5 mM α-methylornithine or 5 mM α-difluoromethylornithine in a 150 cm^2 Costar flask. In another experiment α-difluoromethylornithine (5 mM) was added 23 hr after seeding of cells into fresh growth medium, i.e. after the initial stimulation of polyamine synthesis. Before addition to the cultures, the inhibitors were dissolved in fresh growth medium, neutralized (pH 7.4) and the solutions sterile filtered. The cell number was originally counted in a hemocytometer, but since there was no clumping of cells, a Coulter counter was used for routine counts. At various times after seeding, samples were taken for cell counting, enzyme assay and polyamine analysis. Cells intended for biochemical analyses were harvested by centrifugation and the pellet was stored at -70°C.

The cells were disrupted by sonication in 10 mM Tris-HCl buffer (pH 7.2) containing 5 mM dithiothreitol and 0.05 mM pyridoxal 5'-phosphate. The ornithine decarboxylase activity was assayed for in the presence of a 1 mM L-ornithine concentration essentially as described by Jänne and Williams-Ashman (11). Polyamines were determined according to the method described by Seiler (12). Since pyridoxal 5'-phosphate interferes with the polyamines in the previously used elution medium (13), we used chloroform/isopropanol (25:1) in the present study for development of the thin-layer chromatographic plates. The data was corroborated by analysis of some representative samples on a Durrum D-500 amino acid analyzer (14), kindly made available to us by Dr. L.J. Marton.

Growth medium components were purchased from Gibco Bio-Cult, Paisley, Scotland. DL-α-methylornithine monohydrochloride monohydrate and DL-α-difluoromethylornithine monohydrochloride monohydrate were generously donated by the Centre de Recherche Merrell International, Strasbourg, France.

RESULTS AND DISCUSSION

Figure 1 A-E shows the effect of α-methylornithine on growth and polyamine metabolism of Ehrlich ascites tumor cells in culture. Despite the fact that α-methylornithine has been found to markedly inhibit growth of rat hepatoma cells (2, 3) and mouse L1210 cells (4) at the same concentration (5 mM), it did not affect the growth pattern of the Ehrlich ascites tumor cells (Fig. 1A).

Even though α-methylornithine is a potent competitive inhibitor of ornithine decarboxylase (2, 9), the activity of the enzyme, as assayed *in*

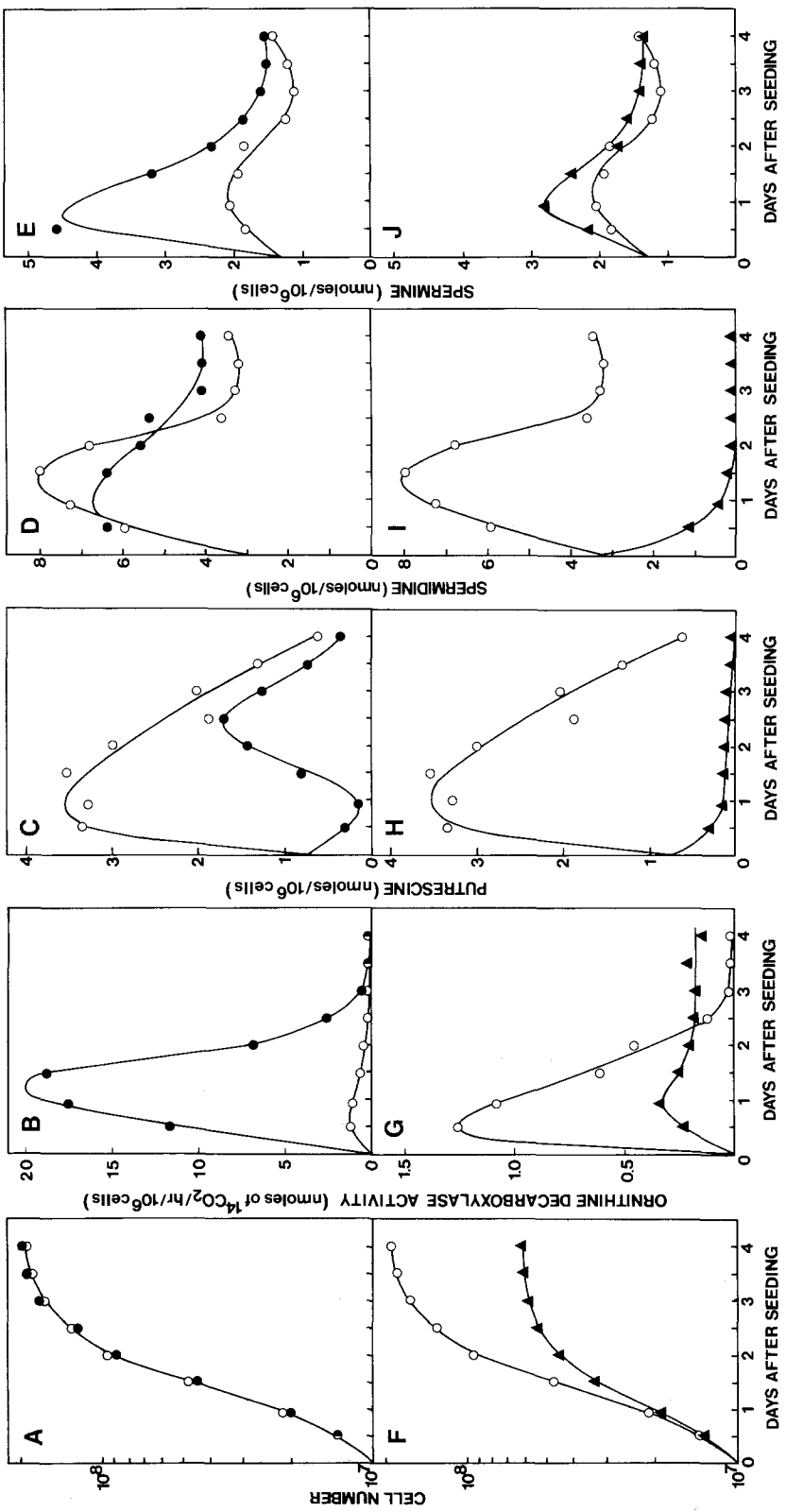

Figure 1. Effect of α-methylornithine (A-E) and α-difluoromethylornithine (F-J) on Ehrlich ascites tumor cell growth and polyamine metabolism. The ornithine decarboxylase inhibitors were added at time 0 to a final concentration of 5 mM. Untreated control cultures (○); cultures treated with α-methylornithine (●) or α-difluoromethylornithine (▲).

vitro, was found to be elevated by as much as 30-fold in α-methylornithine-treated cells (Fig. 1B). The *in vitro* assay technique involved a 700-fold dilution step, which implies that the inhibitor concentration in the reaction mixture amounted to 8 μM at the most. Since α-methylornithine is a reversible inhibitor (K_i = 20-40 μM) (2, 9, 15) that produces a concomitant stabilization of the enzyme (16), the dilution relieves the inhibition that occurs in the cell and liberates the accumulated amount of enzyme. That the intracellular ornithine decarboxylase activity was inhibited, at least initially, is apparent from Figure 1C. Thus, putrescine, which is the product of the reaction, showed a marked decrease in its concentration during the first day of treatment. Subsequently, however, the cellular putrescine content increased and almost reached the control level after 60 hr. Then it decreased in parallel with the control. The increase in cellular putrescine content that occurred after 36 hr of treatment may be attributed to the 30-fold increase in the amount of ornithine decarboxylase; α-methylornithine being unable to suppress all this enzyme activity. Furthermore, complete inhibition of ornithine decarboxylase is likely to be counteracted by the rapid synthesis of new enzyme molecules, which may be catalytically active for a brief period. In fact, the turnover rate (i.e. the rate of both synthesis and degradation) of ornithine decarboxylase may be as short as 5 min (17).

The initial increase in cellular spermidine content occurring after seeding was not markedly affected by α-methylornithine treatment (Fig. 1D) even though putrescine formation was reduced. This fact probably contributed to the depletion of the cellular putrescine content (Fig. 1C). With the depletion of the substrate (putrescine) there was a slight decrease in the rate of spermidine formation, resulting in a lower peak level in the α-methylornithine-treated cells (Fig. 1D). The subsequent decrease in spermidine content was less marked in α-methylornithine-treated cells than in untreated control cells.

During the first day of α-methylornithine treatment there was a marked increase in the cellular spermine content and it remained elevated, compared

to the control level, during the rest of the treatment period (Fig. 1E). This observation may be explained by the fact that putrescine inhibits spermine synthase by competing with spermidine (18). When the putrescine content decreases as a result of α-methylornithine treatment, spermidine (the level of which is not markedly decreased compared to that of the control) competes more favorably for the active site, thus producing an increased rate of spermine formation (Fig. 1E).

A possible explanation for the fact that α-methylornithine inhibits growth of rat hepatoma cells and mouse L1210 cells (2-4), but not that of Ehrlich ascites tumor cells, is that the latter have a putrescine and a spermidine content that is considerably higher than that of the other cells. For human prostate adenoma cells, which were not particularly responsive to α-methylornithine treatment (4), no polyamine concentrations were reported.

Figure 1 F-J shows the effect of 5 mM α-difluoromethylornithine on growth and polyamine metabolism of Ehrlich ascites tumor cells in culture. At variance with α-methylornithine, α-difluoromethylornithine proved to be an effective inhibitor of growth (Fig. 1F). In the presence of the inhibitor, there was a continuous decrease in the growth rate, beginning approximately 1 day after seeding, i.e. when the cells had traversed almost two cell cycles (Fig. 1F). On day 1 (exponential phase) there were 1.9×10^7 and 2.1×10^7 cells and on day 4 (plateau phase) there were 7.5×10^7 and 1.9×10^8 cells in α-difluoromethylornithine-treated and control cultures, respectively. That rat hepatoma cell growth was affected by the inhibitor already after one cell cycle (3, 4) may be due to the presence of a lower initial putrescine content in these cells.

Even though α-difluoromethylornithine is an irreversible inhibitor of ornithine decarboxylase (10), there was no complete inhibition of the enzyme activity (Fig. 1G). This is probably due to the high rate of turnover of the enzyme (17). It is notable that during the plateau phase, when the ornithine decarboxylase activity of control cultures became nondetectable, there was

a residual ornithine decarboxylase activity in α-difluoromethylornithine-treated cells (Fig. 1G).

α-Difluoromethylornithine treatment caused a rapid and permanent depletion of cellular putrescine and spermidine (Fig. 1H-I), which is consistent with the results of others (3, 4). This effect is in marked contrast to the 5-fold increase in putrescine content and 3-fold increase in spermidine content occurring in untreated control cultures.

Similar to α-methylornithine, α-difluoromethylornithine produced a significant increase in the cellular spermine content (Fig. 1J) probably by relieving the inhibition exerted by putrescine on spermine synthase (18). The increase in spermine content in α-difluoromethylornithine-treated cells was not as pronounced as that in cells treated with α-methylornithine (Fig. 1E), probably because of the extensive depletion of the substrate (spermidine) (Fig. 1I). In α-methylornithine-treated cells, however, spermidine was continuously available at a high concentration (Fig. 1D).

It is apparent from these experiments that an irreversible inhibition of ornithine decarboxylase (like that exerted by α-difluoromethylornithine)

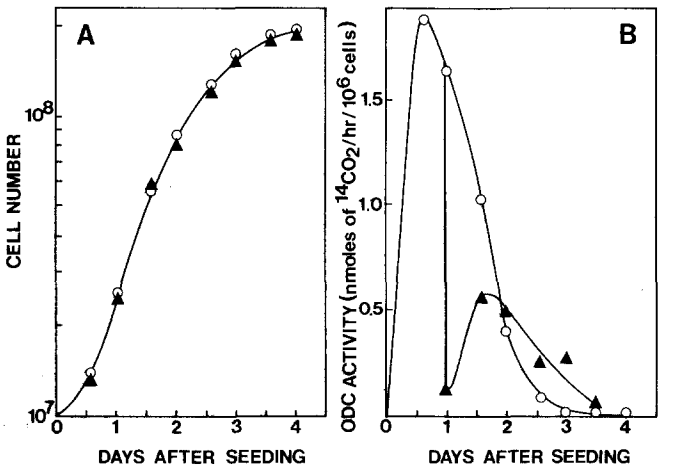

Figure 2. Effect of α-difluoromethylornithine on Ehrlich ascites tumor cell growth (A) and ornithine decarboxylase (ODC) activity (B). α-Difluoromethylornithine was added 23 hr after seeding, i.e. after the growth-stimulated activation of ornithine decarboxylase. Untreated control cultures (○); cultures treated with 5 mM α-difluoromethylornithine (▲).

is required in order to produce an adverse effect on growth of Ehrlich ascites tumor cells in culture. However, when α-difluoromethylornithine was added after the initial surge of ornithine decarboxylase activity (after 23 hr of growth), growth proceeded as in untreated control cultures (Fig. 2). The fact that α-difluoromethylornithine treatment, beginning at 23 hr when the ornithine decarboxylase activity was markedly elevated, only produced a transient decrease in enzyme activity, is probably a consequence of regulatory mechanisms that serve to counteract the development of a cellular polyamine deficiency. A further consequence of such regulatory mechanisms is the maintenance of a high cellular spermine content, despite irreversible inhibition of the initial and rate-limiting enzyme in polyamine synthesis and depletion of the spermine precursors (putrescine and spermidine). This phenomenon is also illustrated by the finding of Mamont et al. (19), that incorporation of ^{3}H-ornithine into putrescine and spermidine was reduced by 98-99 % in α-difluoromethylornithine-treated rat hepatoma cells, whereas the incorporation into spermine was unchanged.

In view of the present data it is apparent that polyamines (at least putrescine and spermidine) are essential for optimal cell growth and division. Consequently, cells that were permitted to expand their polyamine pools, by stimulation of cell proliferation, were found to strongly resist the action of polyamine synthesis inhibitors, even that of an enzyme-activated, irreversible inhibitor of ornithine decarboxylase.

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