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Dihydrofolate reductase induced by folic acid in cultured human cells

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SUMMARY

Cells of human origin in suspension culture increased their dihydrofolate reductase activity during logarithmic growth. High concentrations of folic acid caused a further increase in this activity. The effect of folic acid seemed specific since general protein synthesis and the activity of three other enzymes were not altered in its presence. The folate-induced increase required protein and RNA synthesis. The effects of inhibitors of RNA synthesis suggested that ribosomal RNA, rather than messenger RNA, might be limiting during the induction period.

The enzyme dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of folate and dihydrofolate to tetrahydrofolate by NADPH. Inhibitors of the enzyme increase its level in the cells of patients[1] and in cultured cells of human origin[2], perhaps by stabilizing the enzyme against intracellular degradation[3]. Such inhibitors do not occur naturally so it is of interest that folic acid, a naturally occurring compound, has a similar effect on dihydrofolate reductase activity as we here report.

We grew the human cell line, R.P.M.I. 4265, in suspension culture in R.P.M.I. 1640 medium, containing 10% fetal calf serum, and measured the activity of dihydrofolate reductase during cell growth as reported previously[3]. Cell culture materials were supplied by Grand Island Biologicals. The following compounds were obtained from the sources given: folic acid and formycin, Sigma; actinomycin D (lyovac), Merck, Sharp and Dohme; cycloheximide (Acti-dione), Nutritional Biochemical Co. α-Amanitin was a gift of Dr Cliff Stanners. Dihydrofolate was prepared by reducing folate with dithionite[4].

The rate of protein synthesis was determined by measuring the rate of incorporation of tritium-labelled algal amino acids (New England Nuclear) into acid-insoluble products as described before[3], except that cells were suspended in an amino acid-free medium with dialysed fetal calf serum prior to addition of the label.

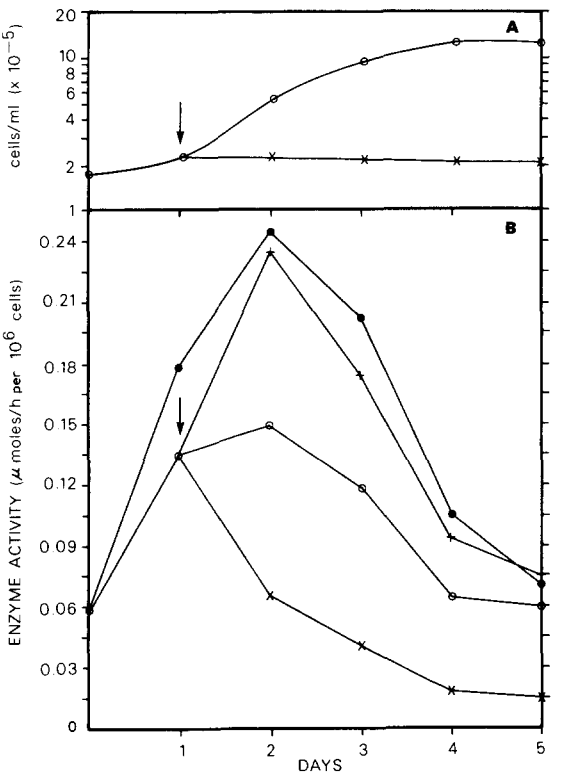

Fig. 1. Dihydrofolate reductase activity following addition of folate and cycloheximide. (A) ○——○, growth curve of cells when folate ($1 \cdot 10^{-4}$ M) was absent or added at zero time or at 24 h (arrow); x——x, growth curve when folate and cycloheximide ($1 \cdot 10^{-3}$ M) were added together (arrow). (B) Variation in enzyme activity under the conditions shown in A: ○——○, control; ●——●, folate added at zero time; +——+, folate added at 24 h (arrow); x——x, folate and cycloheximide added at 24 h (arrow).

In growing cells, dihydrofolate reductase activity increased at 24 h, reached a peak at 48 h during logarithmic growth of the cells, then decreased as they entered a resting state (Fig. 1A and 1B). If folate ($1 \cdot 10^{-4}$ M) was added to the culture at zero time or at 24 h, the growth curve was not affected (Fig. 1A), but an increase in enzyme activity was observed at 48 h, followed by a decrease, almost to control values. When cycloheximide ($1 \cdot 10^{-3}$ M) was added at 24 h with folate, cell growth ceased (Fig. 1A), the increase in enzyme activity was prevented and a decrease followed immediately. If cycloheximide was added at 48 h, 24 h after folate, cell growth was prevented and a decrease in reductase

activity again resulted. The rate of decrease was greater than that in control cells treated with cycloheximide. Actinomycin D, added at 24 h, prevented the increase in enzyme activity observed in control cells at 48 h, but when folate was present as well, the activity was increased by 48%, equal to the percentage increase observed in control cells when folate alone was added (Table I). Formycin also prevented the increase in activity in the absence of folate while in its presence an increase of 100% occurred. An increase of 53% and 47% was observed in the activity per 10^6 cells and in the specific activity, respectively, following treatment of the control cells with α-amanitin (Table I).

TABLE I

EFFECT ON INHIBITORS OF RNA SYNTHESIS ON INDUCTION OF ENZYME BY FOLIC ACID

Addition	*Control*	*Formycin*	*Actinomycin D (0.1 μg/ml)*	*α-Amanitin*
0	100%*	36%	32%	153%**
Plus folate ($1 \cdot 10^{-4}$ M)	147%	72%	44%	130%***

*180 nmoles/h per 10^6 cells. For the α-amanitin experiment, 265 nmoles/h per 10^6 cells or 221 nmoles/mg per h.
**384 nmoles/mg per h.
***263 nmoles/mg per h.

The rate of protein synthesis was the same for cells harvested at 48 h, with or without folate, as were the activities of malate dehydrogenase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase, within limits of error (see Table II).

TABLE II

PROTEIN SYNTHESIS AND ENZYME LEVELS IN CELLS WITH AND WITHOUT ADDED FOLATE

Protein synthesis expressed as cpm per 10 min per $2 \cdot 10$ cells. Enzyme levels expressed as μmoles/h per 10 cells. DHFR, dihydrofolate reductase; MDH, malate dehydrogenase; LDH, lactate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase.

Addition	*Protein*	*DHFR*	*MDH*	*LDH*	*G6PDH*
0	890	0.275	191	150	3.8
Plus folate ($1 \cdot 10^{-4}$ M)	1110	0.470	179	155	4.3

An increase in dihydrofolate reductase activity induced by folic acid in mammalian cells has not been reported previously although an increase has been reported after the use of antifolate agents which bind to the enzyme[1,2]. Folic acid itself did not have this effect in human subjects[1]. The concentration of folic acid used in our study ($1 \cdot 10^{-4}$ M) was approximately 40 times that normally present in the culture medium and 2000 times that in human serum, so the failure to induce the enzyme in human subjects

may have resulted from a failure of the administered folate to achieve high enough concentration *in vivo*. Chello and Bertino[5] have found that destruction of folate in the medium of cultured cells by a carboxypeptidase resulted in a decrease in dihydrofolate reductase activity. This observation and ours are consistent with a role of folate in maintaining intracellular reductase levels.

The observed increase in enzyme activity following folate treatment required protein synthesis, since cycloheximide prevented such an increase. This excluded a post-translational site of action of folate, *e.g.* stabilization of the enzyme, unless cycloheximide, in addition to inhibiting protein synthesis, also prevented degradation of the enzyme, as has been seen with tyrosine transaminase under certain conditions[6].

The interpretation of the results following inhibition of RNA synthesis is not clear. Actinomycin D, at the concentrations used, inhibits the synthesis of ribosomal, messenger and other RNA species. On the other hand, α-amanitin, in chick embryo fibroblast cells, initially stimulates the synthesis of RNA, presumably that synthesised by RNA polymerase I (ref. 7), while formycin inhibits the attachment of poly(A) to mRNA and hence its export to the cytoplasm[8]. Both actinomycin D and formycin had similar effects in preventing the normal increase of enzymic activity seen during cell growth, presumably due to inhibition of protein synthesis by preventing mRNA production. However, folate still increased the enzyme activity, in the presence of these two inhibitors, perhaps by an effect on residual enzyme synthesis. The effects of these inhibitors requires further investigation, particularly the unexpected increase in enzyme activity following α-amanitin.

Brade *et al.*[9] have shown that folate does, in fact, stimulate rRNA synthesis 3-fold in rat kidney. Such an effect was preceded by a 4-fold increase in protein synthesis and an increased activity of several enzymes. In our system, however, overall protein synthesis was not increased in the presence of folate. As well, the specific activity of dihydrofolate reductase increased, and there was no change in the activities of three other enzymes after addition of folate. The effect we observe thus seems to be specific but the mechanism awaits further studies.

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