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ENHANCEMENT AND DEPRESSION BY INOSINE OF THE GROWTH INHIBITORY ACTION OF 5-FLUOROURACIL ON CULTURED JENSEN TUMOR CELLS

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SUMMARY: Continuous exposure of Jensen tumor cells in vitro to 1 mM inosine following a 1 hr exposure to 5-fluorouracil resulted in a 5-fold potentiation of growth inhibition. This effect was abolished by the simultaneous presence of 1 mM cytidine and was attributable to altered metabolic processing of drug anabolites after the uptake of 5-fluorouracil had ceased. In contrast, antagonism to 5-fluorouracil was seen when the cells were exposed successively to 1 mM inosine for 1.5 hr and to 5-fluorouracil for 1 hr. In this case the inhibitory action of the drug was diminished by nearly one-half. Quantitation of the potentiation and antagonism was based upon growth delays measured from growth curves obtained by serial photomicrography.

The cytotoxic action of FUra[3] depends upon its conversion to nucleotides, principally 5-fluorodeoxyuridylate, an inhibitor of dTMP synthetase, and/or 5-fluorouridine 5'-triphosphate, which is utilized as a substrate in the formation of functionally defective fluorinated RNA (1-4). Three separate pathways for the conversion of FUra to nucleotides have been demonstrated, which together include PRPP, ribose-1-P, deoxyribose-1-P and ATP as intermediate cosubstrates (1, 2). It has been shown with cultured mammalian

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[3]Abbreviations: FUra, 5-fluorouracil; PRPP, 5-phosphribosyl-1-pyrophosphate; BES, N,N-bis [2-Hydroxyethyl]-2-aminoethanesulfonic acid; TES, N-tris [Hydroxymethyl] methyl-2-aminoethanesulfonic acid; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; CMF, cell multiplication factor.

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cells that naturally-occurring purine derivatives can cause selective changes in the intracellular pool sizes of these cosubstrates, alter the uptake of FUra into nucleotides and RNA, and correspondingly modify the biological effects of FUra (5-12).

Heretofore, either enhancement of the cytotoxicity of FUra, or protection from it, respectively, has resulted from exposure of one cell line or another to naturally-occurring purine derivatives (7-12). In the present report we show that inosine can potentiate or antagonize growth inhibition by FUra in a single cell type, depending upon the timing of exposure of the cells to inosine relative to a 1 hr exposure to FUra.

MATERIALS AND METHODS

Materials. Newborn calf serum was obtained from Irvine Scientific, Santa Ana, CA, and trypsin (1-300, hog pancreas) was purchased from Nutritional Biochemicals Corporation, Cleveland, OH. All chemicals used were either of analytical grade from commercial sources or of the highest purity obtainable from Sigma Chemical Co., St. Louis, MO, or Calbiochem-Behring Corporation, La Jolla, CA.

Cells and Culture Conditions. Jensen tumor cells were the kind gift of Dr. M. K. Patterson, Jr., Samuel Roberts Noble Foundation, Inc., Ardmore, OK. McCoy's 5a medium (13), with glucose and vitamin B_{12} as in the formulation of Hsu and Kellogg (14), was prepared without serum but with the addition of 10 mM each of BES, TES and HEPES buffers (15) and was stored frozen at -20°. Complete medium was prepared by mixing heat-inactivated newborn calf serum (10%) and gentamicin sulfate (50 µg/ml) with freshly thawed medium and sterilizing by filtration. The pH in air of the complete medium at 23° was 7.1. Cells were cultured with 3 ml of complete medium in 60 mm plastic dishes at 36° in a humidified 5% CO_2 - air atmosphere.

Cell Stocks. Monolayers of Jensen tumor cells were harvested prior to reaching confluence by treatment at 36° for 2-3 min with trypsin (0.05%) in EDTA solution (16) containing 0.2% glucose. The detached cells were suspended in growth medium, pelleted by centrifugation, resuspended in growth medium containing 10% glycerol, frozen (17) and stored at -90°.

Transient Drug Exposure and Cell Growth Curves. Dishes were inoculated from thawed cell stocks to produce tumor cell monolayers growing logarithmically after 3-4 days of incubation. Exposure to FUra was carried out by replacing medium with FUra-containing medium and incubating for selected time periods. Drug exposure was terminated by triple rinsing with medium. Controls were subjected to parallel treatment using drug-free medium. The cells were harvested, suspended in medium, counted and diluted to 40,000 cells/ml. Three-ml aliquots from the stirred cell suspension were pipetted into duplicate dishes, each of which had been scored to present four relocatable microscopic fields. Following 3 hr of incubation, unattached cells were removed by rinsing with medium and aspiration. Medium was replaced and time zero photomicrographs were made of the marked fields. Medium was renewed and the photomicrographs were repeated at approximately 24 hr intervals. Cells in the fields were enumerated on projections of photomicrographic negatives. We used the ratio of the number of cells in each field at a selected time to the number of cells in that field at time zero, the CMF, to quantitate the cell pop-

ulation in each field relative to its time zero population. Thus, photomicrographs from duplicate dishes at a given time provided eight CMFs from which a mean ± standard error was computed. Growth curves were obtained by plotting the latter vs time from a value of 1 at time zero

RESULTS

Growth Delay after Transient Exposure to FUra. Growth curves of Jensen tumor cells after a single 30 min exposure to FUra (5-80 μg/ml) are shown in Fig. 1. The drug under these conditions caused a temporary slowing of the proliferation rate. Growth delay was lengthened with each increase in drug concentration. This action of the drug was expressed quantitatively in terms of a growth-delay factor defined as the ratio of the time intervals required for treated and control cell populations to attain a CMF of 15. Drug-exposed cell populations had re-established normal growth rates by the time they had proliferated to that level (Fig. 1)

Correlation of Growth Delay with Exposure Dose of FUra. Growth-delay factors were calculated from the growth curves of Fig. 1 and also from a

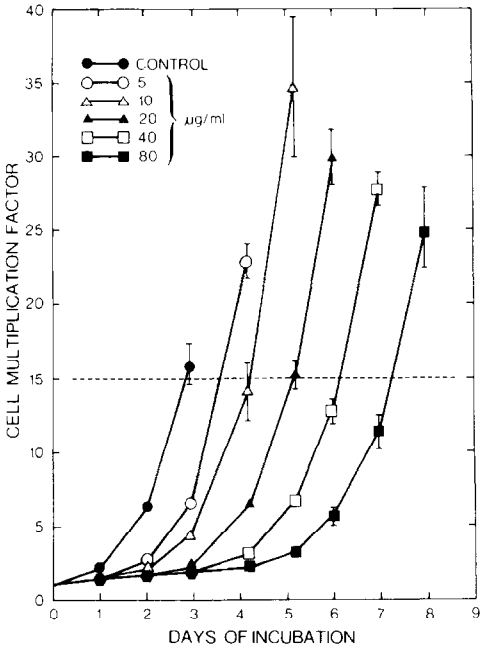

Fig. 1. Growth delay and proliferation of Jensen tumor cells after transient exposure to 5-fluorouracil. Cells in the logarithmic growth phase were exposed to the drug for 30 min, rinsed, harvested and replated at a low density as described in the text. After a 3 hr period of incubation, unattached cells were removed by rinsing and aspiration. Cell proliferation was followed by serial photomicrography. Each point represents the mean of the cell multiplication factors obtained from eight marked microscopic fields. Bars, S.E. Symbols without bars overlie S.E.

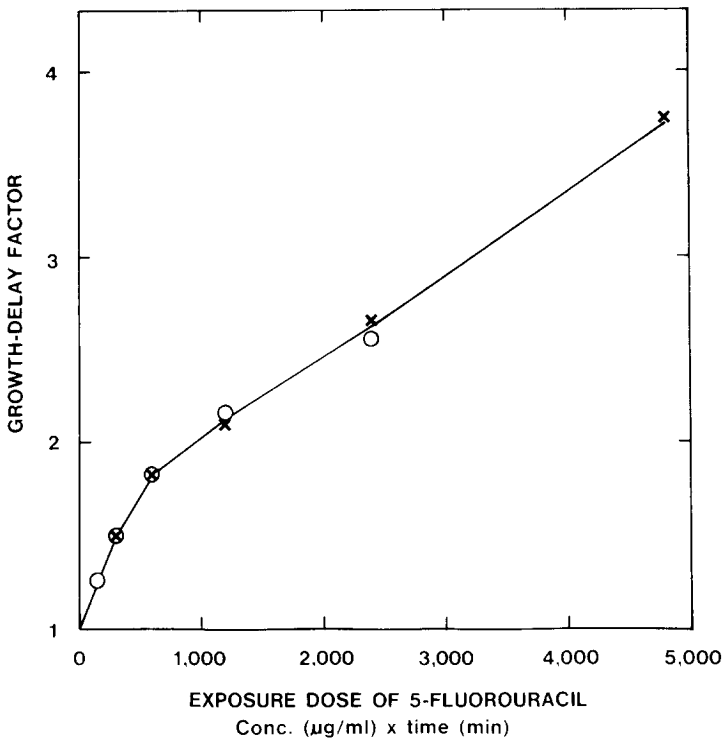

Fig. 2. Correlation of growth delay with concentration of 5-fluorouracil and treatment time. Growth-delay factor: the ratio of the time intervals required for drug-treated and control cell populations to increase 15-fold. Exposure time: 30 min (○); 60 min (×).

similar set of growth curves obtained following a 60 min exposure of cells to FUra (5-80 µg/ml). These values were plotted against drug exposure doses expressed as the product of FUra concentration and treatment time as shown in Fig. 2. The points on the curve reveal that identical exposure doses derived from different combinations of FUra concentration and exposure time give rise to essentially the same growth-delay factor. Thus growth delay is tightly correlated with drug exposure dose and can be utilized to assay FUra exposure doses or their equivalents by means of a standard curve (Fig. 2).

Potentiation by Inosine of Growth Inhibition by FUra and its Reversal. When cells previously exposed to FUra (8.0 µg/ml) for 1 hr were maintained in medium supplemented with 1 mM inosine, growth delay was more than doubled by comparison with cell cultures similarly treated but maintained in medium lacking inosine (Fig. 3A). Cells exposed to 1 mM inosine without prior exposure to FUra exhibited a relatively small growth delay. It is noteworthy that the latter was diminished by addition of 1 mM cytidine (Fig. 3A). Poten-

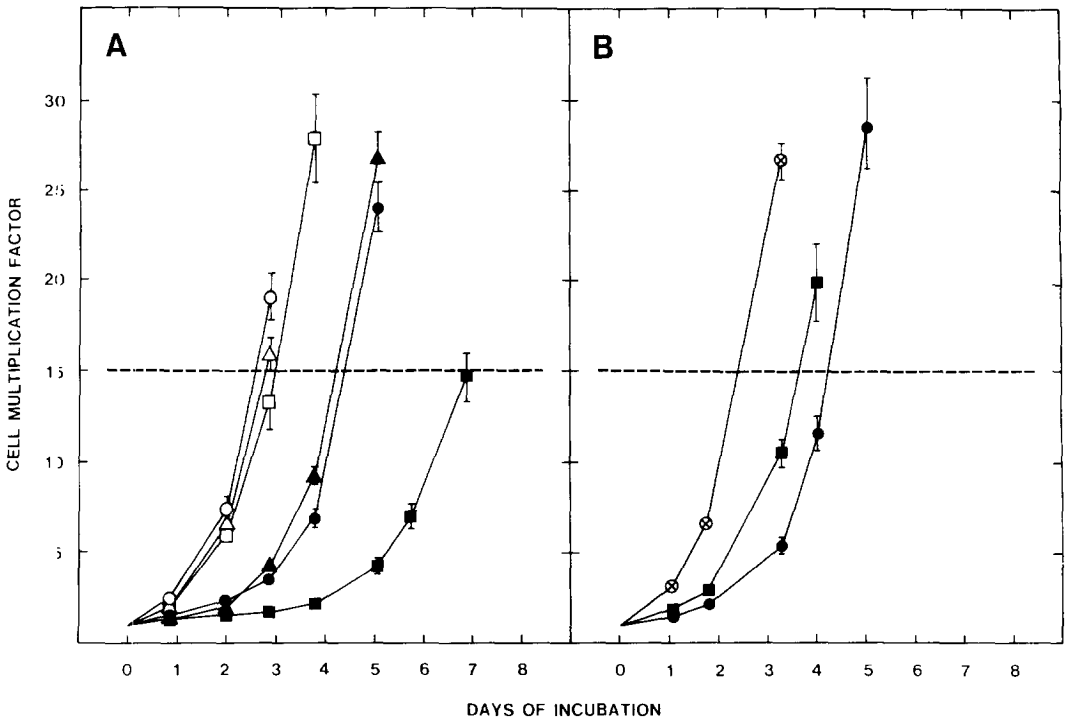

Fig. 3. Modulation by inosine of the growth inhibitory action of 5-fluorouracil on Jensen tumor cells. Cell proliferation was followed by serial photomicrography. Bars, S. E. Symbols without bars overlie S. E. A, Potentiation. Cells were treated for 1 hr by replacement of medium, then harvested, replated as described in the text, and incubated in selected media. Open symbols: treatment with FUra omitted; incubated in drug-free medium (○), medium containing 1.0 mM each of inosine and cytidine (△), and medium containing 1.0 mM inosine (□). Closed symbols: cells treated with FUra (8.0 μg/ml); incubated in drug-free medium (●), medium containing 1.0 mM each of inosine and cytidine (▲), and medium containing 1.0 mM inosine (■). B, Antagonism. Cells were treated, rinsed, and treated again by replacement of medium. The cells were then harvested, replated and incubated in drug-free medium as described in the text. Treatments were as follows: drug-free medium 1.5 hr and 1 hr (○); 1.0 mM inosine 1.5 hr and drug-free medium 1 hr (✕); 1.0 mM inosine 1.5 hr and FUra (8.7 μg/ml) 1 hr (■); drug-free medium 1.5 hr and FUra (8.7 μg/ml) 1 hr (●).

tiation by inosine was abolished if 1 mM cytidine was present concomitantly with inosine in the medium (Fig. 3A). Cytidine exhibited a slight tendency to interfere with growth inhibition by the active anabolites of FUra (Fig. 3A).

Quantitation of Potentiation by Inosine. From Fig. 3A the growth-delay factors for inosine, FUra, and FUra followed by inosine were found to be 1.15, 1.67, and 2.65, respectively. These were equivalent to FUra exposure doses of 90, 480, and 2,480 $\mu g \cdot ml^{-1} \cdot$ min (Fig. 2). Subtracting the small contribution to the inhibition made by inosine alone, i.e., 90 $\mu g \cdot ml^{-1} \cdot$ min, the net po-

tentiating effect of 1 mM inosine was to increase the effectiveness of the exposure dose of FUra from 480 to 2,390 $\mu g \cdot ml^{-1} \cdot$ min, a 5.0-fold enhancement. In similar experiments with 0.5 mM inosine we found a 3.1-fold enhancement, which showed that potentiation by inosine was concentration-dependent.

Inosine-Induced Antagonism to FUra. Exposure of the cells to 1 mM inosine for 1.5 hr, followed by exposure to FUra (8.7 μg/ml) for 1 hr in the absence of inosine, resulted in a marked shortening of growth delay (Fig. 3B). Calculated as before, the effectiveness of the exposure dose of FUra was lowered by inosine from 520 to 300 $\mu g \cdot ml^{-1} \cdot$ min, a reduction of 42 percent.

DISCUSSION

A new aspect of modulation is revealed in the capability of inosine to potentiate growth inhibition by FUra when added to the cells after the uptake of FUra has ceased. Previously, potentiation or antagonism by naturally-occurring purine derivatives has been attributed to stimulation or inhibition of the metabolic activation of FUra (7-12). Now it appears that inosine can act by modifying the intracellular processing of anabolites of FUra already present, altering their distribution and/or their retention. The fact that cytidine prevents this mode of potentiation by inosine suggests that the potentiation is mediated by a decrease in the size of the endogenous pyrimidine nucleotide pool. The observed partial reversal by cytidine of the growth delay caused by inosine alone is in accord with this view. Exposure of mammalian cells to inosine can deplete intracellular PRPP, probably by purine salvage enzymes acting on hypoxanthine derived from inosine (8,11,18). Furthermore it has been demonstrated that a critical lowering of the cellular PRPP concentration inhibits *de novo* pyrimidine nucleotide synthesis and decreases the pyrimidine nucleotide pool (6,8), most likely by retarding the conversion of orotate to orotidylate (12,19) and by reducing the rate of synthesis of carbamyl phosphate (19).

The protection against FUra afforded by a 1.5 hr preincubation of Jensen tumor cells with 1 mM inosine implies a reduced metabolic activation of FUra during its uptake period. In lymphomatous mouse T-cells maximal PRPP lowering occurs after a 1.5 hr exposure to inosine (8). Moreover, the conversion of FUra to nucleotides *via* the nucleoside phosphorylase and nucleoside kinase pathway becomes inactive when inosine, as a source of ribose-1-P, is deleted from the growth medium of a line of hepatoma cells (7). Accordingly, Jensen tumor cells may activate FUra by the PRPP pathway in the absence of inosine, and preincubation with inosine may protect against FUra by depleting PRPP.

Further investigation to explore the biochemical correlates of our experimental results is in progress.

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