Levels of Thymidylate Synthetase during Normal Culture Growth of L1210 Cells

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ABSTRACT

The binding of 5-fluoro-2'-deoxyuridylate generated from 5-fluoro-2'-deoxyuridine in intact cells was used to measure changes in the level of thymidylate synthetase during the course of population growth of murine leukemia L1210 cells. By the use of elution techniques and flow cytometric analysis, the amount and activity of thymidylate synthetase associated with the various phases of cell cycle were determined for the L1210 cells during unperturbed in vitro culture growth. Fluctuations of thymidylate synthetase levels were associated with the cell cycle; there was a positive correlation (P < 0.001) between the percentage of the total cell population in S phase and the concentration of thymidylate synthetase, although there was an increase in the level of this enzyme in association with an increase in G2-M cells, this did not achieve statistical significance. A negative correlation between G1 cells and the concentration of thymidylate synthetase was also observed. The maximum amount of thymidylate synthetase was nearly 900 fmol/10^6 cells and occurred in cell populations during logarithmic growth when the percentage of the population in S phase and G2-M phase was greater than 50 and 20%, respectively. In late culture growth (plateau) when only 25% of the cell population was in S phase and nearly 75% of the population was in G1 phase, the level of enzyme was reduced to 200 fmol/10^6 cells.

INTRODUCTION

DNA replication during S phase of the eukaryotic cell cycle is dependent on the availability of deoxynucleoside triphosphates. TMP synthetase is essential for the synthesis of TMP and therefore TTP. Numerous investigators have evaluated the relationship among the amount of this enzyme, its activity, and the cell cycle (1-5). As with other enzymes associated with DNA synthesis, the concentration or activity of TMP synthetase increases when cellular replication occurs (6-8). In none of these studies, however, were unperturbed cell populations used (6-9). Rode et al. (8) demonstrated that various enzyme activities can differ if measured in whole cells compared to studies using cell extracts. Artifacts may also be introduced by the procedures used to synthesize cells: Colcemid (6); hydroxyurea (8); or some form of nutritive deprivation (7-9). This study attempts to avoid the first of these shortcomings by measuring directly the quantity of TMP synthetase contained within the cells as well as the activity of this enzyme. The second problem, that of cell synchronization, was circumvented by using normally growing cultures of L1210 cells. Correlations of TMP synthetase to the phase of the cell cycle were made by determining the percentage of S, G2-M, and G1 phase cells by flow cytofluorographic methods during the growth of the cell population. Elutriation techniques were also used to isolate populations of cells enriched for cells in specific stages of the cell cycle. We believe, since many biochemical and drug metabolism studies are performed in unperturbed growing cell populations, that this information may be useful to better understand the complexities which can influence experimental results and conclusions.

MATERIALS AND METHODS

Murine leukemia L1210 cells were maintained by three times weekly passages in Fischer's medium (Gibco, Grand Island, NY) supplemented with 10% horse serum (Gibco) in a 5% CO2 incubator at 37°C. Cultures were established at approximately 500 cells/ml and grown in roller bottles containing 500 ml of medium. Dilution resulted in a brief cessation of growth which lasted approximately 6 to 10 h before the population began doubling at a rate of once every 11.9 ± 2.16 (SD) h. Cells which were maintained in plateau phase for greater than 24 h were compared to cells harvested during log phase. Population densities were determined using a Model ZBI Coulter Counter (Hialeah, FL).

Determination of Thymidylate Synthetase Levels. To cell cultures at known population densities incubating at 37°C was added [H]FdUrd (20 Ci/mmol; Moravek Biochemical, City of Industry, CA) to a concentration of 1 μM. Following a 30-min exposure to the radiolabeled FdUrd, the cells were collected by centrifugation at 4°C, washed once with phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4), and lysed by sonication in a buffer of 20 mM NaH2PO4, 10 mM β-mercaptoethanol. FdUrd is maximally accumulated with these cells by 30 s with over 90% being converted to FdUMP. Cell populations enriched for various phases of the cell cycle were used as controls to be certain that drug accumulation was not affected by the different cell populations undergoing study. A 1 μM concentration results in FdUMP levels which exceed the amount of TMP synthetase manymfold (10). Therefore, since FdUMP binds covalently to TMP synthetase in the presence of N-5,10-methylenetetrahydrofolate, we used FdUrd to quantitate TMP synthetase. The [H]FdUMP-TMP synthetase complex was separated from unbound FdUrd by high-performance liquid chromatography over a Toyo Soda TSK G2000SW column (Attex Inst., Berkeley, CA). This method for determining total TMP synthetase has been described in detail by us. The technique excludes non-enzyme-bound radiolabeled FdUrd and other radiolabeled nucleotides (10).

The following experiments were performed to assure that all of the TMP synthetase was bound using this procedure. Cells not given FdUrd were lyzed as before, 1 μM [H]FdUrd and 67 μM N-5,10-methylenetetrahydrofolate were added to form the covalent enzyme complex, and the reaction mixture was incubated at 37°C. The reaction was complete within 5 min and the amount of enzyme bound was the same as that from whole cells given [H]FdUrd. To determine if residual TMP synthetase activity persisted following a 30-min incubation with FdUrd, tritium...
released from [5-3H]deoxyuridine was quantified (11). Enzyme activity could not be detected; therefore in the whole cell system the concentration and exposure period of FdUrd were optimal for maximum binding to TMP synthetase.

Elutriation of Cell Populations. Cell cultures at various phases of population growth (i.e., early log phase, mid-log phase, late log phase) were separated into different subsets based on cell density by the JE-6 elutriator rotor (Beckman Instruments, Palo Alto, CA). Rotor speed was kept constant at 2000 rpm. Cells were collected by flowing Fischer's medium supplemented with 10% horse serum through the rotor beginning at a flow rate of 20 ml/min. Fractions of 25 ml were collected. The flow rate was increased by 5 ml/min after every two fractions. A total of 350 ml were allowed to flow through the rotor. Fraction 1 and occasionally fraction 2 were collected before the elutriation gradient was established. These fractions were discarded and discounted in computing. Fraction 12 was discarded because the rotor was turned off during its collection, but the few cells found in it were included in determining the total of cells fractionated by elutriation.

Determination of Cell Cycle by Flow Cytometric Analysis. Cells were fixed for more than 24 h at 4°C in 10% formalin-90% phosphate-buffered saline. Cells were washed with 4 n HCl for 20 min and with distilled H2O. Cells were stained for 20 min in 0.02% acriflavine HCl (Sigma, St. Louis, MO)-0.05 n HCl. Unbound dye was removed by two washes with acid alcohol (100 ml of 70% ethanol plus 1 ml of concentrated HCl), resuspended in distilled H2O, wrapped in foil, and stored for less than 24 h at 4°C.

DNA content was analyzed by measurement of the fluorescence of acriflavine using a FACS IV (Becton-Dickinson, Sunnyvale, CA). Percentages of cells in various phases of the cell cycle were determined by the equations derived by Dean and Jett (12). Although there are some weaknesses in their descriptive properties, these equations are well suited for the fitting of staining data in unperturbed cell populations.

Statistical Analysis. Correlations between levels of TMP synthetase and cell populations in various phases of growth were made using linear regression analysis and nonparametric Spearman rank order correlation programs on an HP-67 calculator (Hewlett Packard, Corvallis, OR).

RESULTS

TMP Synthetase Levels during the Growth of the L1210 Cell Population. Cultures were grown from low population density through the logarithmic and into plateau phase. The amount of TMP synthetase per cell remained essentially constant from the onset of the cell population growth at low cell densities to the higher cell densities near the middle of the logarithmic phase. A decrease in the amount of TMP synthetase per cell was observed only during the period of growth from midlogarithmic to plateau cell densities; cells in plateau phase growth had the lowest TMP synthetase levels (Fig. 1).

Correlations between Cell Cycle and TMP Synthetase. At each point where TMP synthetase was measured, an aliquot of cells was removed and analyzed by the FACS IV for distribution of the cell cycle. The results are also presented in Fig. 1. There was a positive correlation between levels of TMP synthetase and percentage of cells in S phase with correlation coefficients (r) of <0.9 for linear regression. The data were also analyzed using nonparametric Spearman rank ordering. Correlations using this test were 0.89. To corroborate this observation, cell populations underwent elutriation to segregate the cells into various subsets based on cell size and density and the S, G2-M, and G1 phases and TMP synthetase were determined and correlated.

TMP Synthetase Levels in Cells Collected by Elutriation. Cell culture populations at three cell concentrations obtained during normal cell growth (45,000, 113,000, and 1,054,000 cells/ml) were evaluated by elutriation (Table 1). These cell densities correspond to the appropriate point of growth in Fig. 1. The indicated samples were split: on one-fourth of the sample the TMP synthetase levels were determined; and on the remaining three-fourths, the percentage of cells in the phases of cell cycle were measured.

There was a negative correlation between G1 phase and levels of TMP synthetase. Both linear regression analysis (r = 0.825) and Spearman's rank correlation (rs = 0.818) yielded P values of <0.001. Positive correlations between S phase and TMP synthetase were also evident: p < 0.01. A similar trend was noted for G2-M cells and the level of TMP synthetase, but this did not reach statistical significance.

DISCUSSION

Previous reports have documented changes in TMP synthetase activity in artificially synchronized cells that ranged from 4-fold in Hepa mouse hepatoma cells (9) to 60-fold in L1210 cells (8). The current study demonstrates that changes within L1210 cell populations were only 4-fold between cells in logarithmic phase and those in plateau phase growth. If the agents used in the previous studies to arrest the passage of the cells through the cycle altered the rate of synthesis or the catabolism of TMP synthetase, misleading enzyme levels or activity could have resulted. This may explain why the fluxes previously measured varied so greatly.
Calculation of TMP synthetase in cells within the various phases of the cell cycle

From the data in Table 1, the calculation was made for the amount of TMP synthetase contained in cells of the G1, S, and G2-M portion of the cell cycle. This was accomplished by solving simultaneous equations for three variables for each cell concentration.

<table>
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<th>Cell concentration (x10^4 cells/ml)</th>
<th>Sample fractions pooled</th>
<th>% of total cells</th>
<th>% in phase of cell cycle</th>
<th>FdUMP binding (fmol/10^6 cells)</th>
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Table 1

Calculation of TMP synthetase in the various cell fractions obtained from elutriated cell populations of L1210 cells

The thymidylate synthetase quantity was measured by isolation of the covalently bound FdUMP-enzyme-folate complex formed in the presence of [3H]FdUrd. From the data in Table 1, the calculation was made for the amount of TMP synthetase contained in cells of the G1, S, and G2-M portion of the cell cycle. This was accomplished by solving simultaneous equations for three variables for each cell concentration.

Calculation of FdUMP binding in cells within the various phases of the cell cycle

From the data in Table 1, a calculation was made for the amount of FdUMP binding in cells of the various phases of the cell cycle. This was accomplished by solving simultaneous equations for three variables for each cell concentration.

**unperturbed cell populations; therefore, at any time when the TMP synthetase levels and activity were determined, there were always many cells included which were distributed in all phases of the cell cycle. The maximum percentage of cells in S phase approximated only 50% in early cell growth. Therefore, the amount of TMP synthetase present in those cells in S phase could in fact be greater than our values would indicate. We do show that a positive correlation does exist between S phase and this enzyme. This, of course, is not surprising because of the need of TTP for DNA synthesis which occurs during the S phase of the cell cycle. The nature of the mechanism by which these cell cycle-specific changes in TMP synthetase levels occur is only speculative but may include regulation by product inhibition (8) or allosteric interactions with other enzymes involved in DNA replication (13).

In a population of L1210 cells, the average cell volume is reduced by approximately 40%, when the population reaches plateau growth (14). This volume reduction could result in a total reduction in enzyme and yet maintain a steady state intracellular concentration; however, the magnitude of the TMP synthetase levels and a negative association between G1 and S phase cells. Therefore, there are always many cells included which were distributed in all phases of the cell cycle.

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