MOLECULAR PHARMACOLOGY, 16, 601-606

Thymidine Induced Perturbations in Ribonucleoside and Deoxyribonucleoside Triphosphate Pools in Human Leukemic CCRF-CEM Cells

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(Received December 18, 1979)
(Accepted April 23, 1979)

SUMMARY

GRINDEY, GERALD B., MING C. WANG, AND JAMES J. KINAHAN. Thymidine induced perturbations in ribonucleoside and deoxyribonucleoside triphosphate pools in human leukemic CCRF-CEM cells. *Mol. Pharmacol.* **16:** 601-606 (1979).

The inhibition of growth of CCRF-CEM cells in culture by various concentrations of thymidine was correlated with alterations in the intracellular concentration of deoxyribonucleoside triphosphates. With increasing growth inhibition, the concentrations of dTTP, dGTP and dATP all increased while that of dCTP decreased somewhat. Although there was a linear relationship between the decrease in growth rate and the concentration of dCTP, the change in the pool of dCTP does not appear to be of sufficient magnitude to account for the growth inhibition. The addition of deoxycytidine (3 to 10 μM) completely reversed the growth inhibition produced by thymidine. Higher concentrations of deoxycytidine (20 μM), however, again produced a 13% inhibition of growth rate in the presence of thymidine. The intracellular concentrations of dCTP and dATP were equal to control values; those of dTTP and dGTP were increased. The results indicate that small perturbations in the deoxynucleosides triphosphate pools inhibit cellular growth and that increases as well as decreases in these pools may inhibit DNA synthesis as mediated through their interaction with DNA polymerase.

INTRODUCTION

Deoxyribonucleosides, especially thymidine, are toxic to numerous mammalian cells in culture (1-10) and this effect has been attributed to feedback regulation on ribonucleotide reductase by increased pools of deoxyribonucleoside triphosphates. Evaluation of the characteristics of this enzyme isolated from bacterial (11, 12) or mammalian (13) sources indicated that substrate specificity and overall activity are directly controlled by ATP, dTTP, dGTP and dATP. For the mammalian enzyme, dTTP activates the reduction of GDP to dGDP while inhibiting the reduction of pyrimidine nucleotides (13). The observed increase in dTTP and dGTP with decreased levels of dCTP following exposure of synchronized Chinese hamster ovary cells in culture to high concentrations of thymidine led Bjursell and Reichard (8) to conclude that the allosteric mechanisms shown with the purified enzyme operate in intact cells. They also reported that a decline in rate of DNA synthesis was correlated in time with the decrease in dCTP and that both of these effects were reversed by the addition of deoxycytidine to the culture medium (8).

This investigation was supported by Public Health Service Research Grant CA-17156 from the National Cancer Institute.

0026-895X/79/050601-06$02.00/0

Overall, the results suggested the possibility that the size of the dCTP pool may have a regulatory function for controlling the rate of DNA synthesis (8).

More recently, Lowe and Grindey (9) described fluctuations in deoxyribonucleoside triphosphate pools in L1210 cells under conditions of variable inhibition of steady-state growth induced by thymidine. In these studies, there was no direct relationship between the size of the dCTP pool and rate of DNA synthesis. While there appeared to be a direct relationship between the size of the deoxynucleotide pools and growth rate inhibition, the data suggested that the mechanism of thymidine induced inhibition of growth was more complex than simple substrate limitation of DNA polymerase (9). In the present study, the relationship between the concentrations of ribonucleotides and deoxyribonucleoside triphosphates in CCRF-CEM cells and growth rate inhibition induced by thymidine is further evaluated.

MATERIALS AND METHODS

The CCRF-CEM human leukemia suspension culture used in these studies was obtained from DeWayne Roberts (14), St. Jude Children's Research Hospital, and was derived from the blood of a patient with acute leukemia that developed from lymphosarcoma (15). The cells were grown by diluting the suspension to 1×10^5 cells/ml every third day with Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% dialyzed fetal calf serum (Grand Island Biological Co.). For experiments, the medium was also supplemented with the organic buffers N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (20 mM) and morpholinopropane-sulfonic acid (10 mM) (Sigma) to stabilize the PH at 7.3; these buffers did not inhibit the growth of CCRF-CEM cells at these concentrations. Under these conditions, the growth kinetics of CCRF-CEM cells were relatively independent of inoculum density and volume of the culture (16). The exponentially growing CCRF-CEM cells were harvested, extracted, and assayed enzymatically for deoxynucleoside triphosphates, as previously described (9).

RESULTS

CCRF-CEM cells in culture can be grown logarithmically in RPMI 1640 using 10% dialyzed fetal calf serum with a doubling time of 18 hours (16). Under these conditions the cells achieve an 8-fold increase in cell number during 56 hours of incubation. The addition of increasing concentrations of thymidine to the culture medium inhibits the growth of these cells achieving a spectrum of steady-state growth rates (Fig. 1). After 45 hours of incubation, the CCRF-CEM cells were harvested, deoxynucleoside triphosphate pools extracted and the changes in these pools were correlated with the changes in growth rate. As shown in Figure 2, the addition of 5 μM thymidine to the culture medium achieved only a 5% decrease in growth rate; 40 μM thymidine inhibited by 73%. As expected, the intracellular concentration of dTTP increased substantially, reaching eight times the control value at 40 μM thymidine, while the pools of dGTP and dATP also increased 3–5 fold. Only a 42% decrease in the concentration of dCTP was observed in this cell line at a concentration of thymidine that produced a 73% inhibition of growth rate. Alterations in the concentration of ribonucleoside triphosphates induced by thymidine are summarized in Figure 3. The concentration of

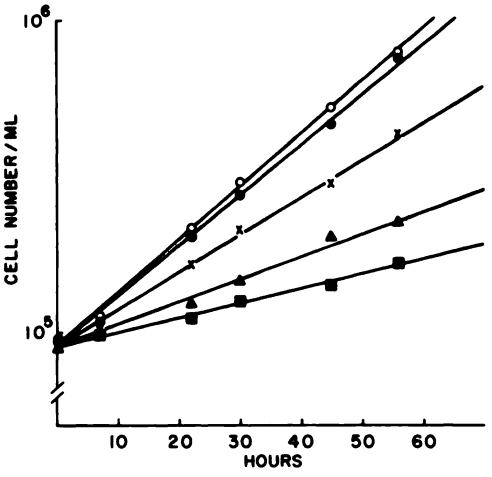

FIG. 1. *Effect of various concentrations of thymidine on the growth kinetics of CCRF-CEM cells*

The symbols are: ○, untreated controls: ●, 5 μM TdR; ×, 10 μM TdR; ▲, 20 μM TdR; and ■, 40 μM TdR.

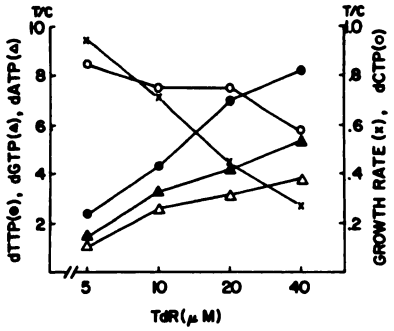

FIG. 2. *Relationship between growth rate inhibition and perturbations induced by thymidine in the deoxyribonucleoside triphosphate pools*

Fractional inhibition of control growth rate was determined by comparison with control slopes after 45 hours of inhibition (Fig. 1), and the cells were assayed at this time. Control values for the deoxynucleotides expressed as picomoles/10^6 cells are: 65, dTTP; 26, dCTP; 53, dATP; and 37, dGTP. The symbols are: T/C, treated/control; ×, growth rate; ○, dCTP; ●, dTTP; ▲, dGTP; and △, dATP.

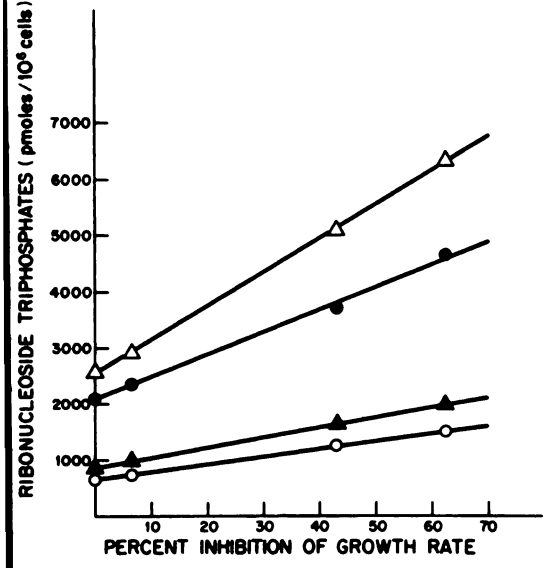

FIG. 3. *Relationship between growth rate inhibition and perturbations in ribonucleoside triphosphate pools induced by thymidine*

The symbols are ○, CTP; ▲, GTP; ●, UTP; and △, ATP.

all four triphosphates increased in direct proportion to the inhibition of growth rate and reached 225–245% of control values at 62% inhibition. For example, CTP levels increased from 663 to 1494 picomoles per 10^6 cells. The concentration of the ribonucleoside diphosphates also increased in a comparable manner.

The toxicity of thymidine against mammalian cells in culture is completely prevented by the addition of deoxycytidine to the culture medium (4, 6, 8, 9). The ability of deoxycytidine to reverse the growth rate inhibition of CCRF-CEM cells by thymidine along with perturbations in the deoxyribonucleoside triphosphate pools is shown in Figure 4. The effects of 32 μM thymidine alone on growth rate and deoxynucleoside pools was evaluated within this experiment for direct comparison. The addition of 2 μM deoxycytidine in combination with 32 μM thymidine almost completely prevented the growth rate inhibition (98% of control) and increased the dCTP pools up to control values. Moreover, there was a substantial decrease in the pools of dTTP, dATP and dGTP with dATP returning to control values. At 3 μM deoxycytidine, no growth rate inhibition was detected and the dCTP pools remained constant. The pools of the other deoxynucleotides decreased slightly. The addition of higher concentrations (20 μM) of deoxycytidine in combination with thymidine again resulted in a 13% inhibition of growth rate with no detectable change in the pools of dCTP. The pools of dTTP and dGTP were slightly increased over those found under conditions of full reversal of the growth rate inhibition produced by thymidine. This unexpected inhibition of growth and pool size changes was confirmed in a separate experiment.

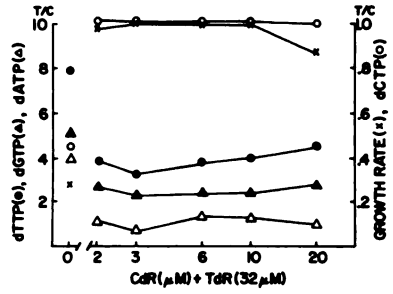

FIG. 4. *Relationship between growth rate inhibition and induced perturbations in the deoxynucleoside triphosphate pools by combinations of thymidine and deoxycytidine*

The experimental conditions and symbols are described in the legend to Figure 2.

The four ribonucleoside triphosphate pools each decreased with deoxycytidine addition and remained only slightly above control values at 20 μM deoxycytidine (data not shown). Higher concentrations of deoxycytidine did not produce any additional growth rate inhibition in the presence of thymidine.

DISCUSSION

Previous studies in L1210 cells relating growth rate inhibition induced by thymidine or deoxyguanosine and deoxyribonucleoside triphosphate pools indicated a relationship between these pools and the rate of DNA synthesis (9). Growth inhibition by thymidine resulted in increased pools of dTTP and dGTP, while dCTP and dATP pools decreased somewhat. Deoxyguanosine inhibition of growth was associated with decreased dTTP and dCTP pools, unchanged dATP pools and elevated concentrations of dGTP. However, the results indicated that the relationship between these pools and DNA synthesis was more complex than simple substrate limitation of DNA polymerase; the decreases in limiting pools were not of sufficient magnitude to account for the observed cell growth inhibition (9).

In this study using CCRF-CEM cells, thymidine inhibition of growth resulted in increased pools of dTTP, dGTP and dATP while that of dCTP decreased somewhat (Fig. 2). Under these conditions, only moderate increases in the ribonucleotide pools were observed (Fig. 3). Although the decrease in dCTP was in direct proportion to growth rate inhibition, the changes appear too small to account for this inhibition, even assuming that only the pool of dCTP is limiting for DNA synthesis (Fig. 2). Assuming that dCTP is limiting for DNA synthesis, and below K_m, and that the system behaves in a Michaelis-Menton fashion, then a 50% reduction in dCTP should produce only 50% decrease in the rate of DNA synthesis. At dCTP concentrations at or above K_m, an even larger decrease in the dCTP pool would be required to produce a comparable inhibition. Using a similar approach to data analysis, Nazar *et al.* (17) were able to conclude that ribonucleoside triphosphate pools were not limiting for RNA polymerase activity in *E. coli*. Thus, the reduction in dCTP pools from 53 picomoles per 10^6 cells to a level of 31 picomoles per 10^6 cells seems insufficient to account for a 73% reduction in growth rate. Because the other deoxynucleoside triphosphate pools all increased, such an analysis also indicated that the increased deoxynucleoside triphosphate pools may be partially responsible for the observed growth rate inhibition.

Alterations in deoxyribonucleoside triphosphate pools under conditions of reversal of the thymidine induced inhibition of growth by deoxycytidine lends additional support to this proposal (Fig. 4). While addition of deoxycytidine to the culture medium increased the pool of dCTP back to control values, a dramatic decrease in the concentrations of dTTP, dGTP and dATP was also observed. In CCRF-CEM cells, the addition of 20 μM deoxycytidine in combination with thymidine results in 13% inhibition of growth rate (Fig. 4). Under these specific conditions, the concentrations of dCTP and dATP are identical to control values, while the pools of dTTP and dGTP are both increased over control. Thus this growth rate inhibition cannot be attributed to a depletion in the concentration of any of the deoxynucleoside triphosphates. This effect of deoxycytidine was not observed in similar studies using L1210 cells in culture (9).

Lowe *et al.* (10) recently evaluated the effects of deoxyadenosine on nucleotide pools and cellular cytotoxicity using L5178Y cells in culture. The significant changes in the intracellular concentration of deoxynucleoside triphosphates during substantial growth inhibition were an increase in dATP and a decrease in dCTP. The addition of deoxycytidine to the growth medium along with the deoxyadenosine resulted in dCTP, dTTP and dGTP levels that were comparable to control values. However, the increase in dATP and growth inhibition were not altered. Thus Lowe *et al.* (10) concluded that this inhibition resulted from increased pools of dATP rather than reduced pools of deoxynucleoside triphosphate required for DNA synthe-

sis. These overall results indicate that increases in the intracellular concentration of either dTTP or dATP may inhibit DNA synthesis, but the concentrations of the other deoxynucleotides are also of importance. Inhibition of growth by deoxyguanosine results in an increase in only dGTP while dTTP and dCTP pools are decreased somewhat (9).

The potential mechanism for inhibition of DNA synthesis by deoxynucleoside triphosphates has recently been identified (18)[1]. A regulatory protein for DNA polymerase α responsive to deoxyribonucleoside triphosphates has been isolated from calf thymus. In the presence of this protein, the activity of DNA polymerase α was inhibited by dGTP in a concentration dependent manner achieving 98% inhibition at 200 μM dGTP compared to virtually no inhibition of polymerase activity in the absence of this protein (18). Kinetic studies using homopolymers as template with calf thymus DNA polymerase in the presence of this regulatory protein have demonstrated that the other deoxynucleoside triphosphates are also inhibitors of polymerase activity in addition to their substrate function[1]. Although the decreased dNTP pools associated with thymidine or deoxyguanosine toxicity were not of sufficient magnitude to account for the observed inhibitions, the increased pools of either dTTP or dGTP are of sufficient magnitude to provide substantial inhibition of DNA polymerase α in the presence of these regulatory properties.

Such a regulatory mechanism for DNA polymerase is capable of potentiating the decrease in DNA synthesis that would be expected from a given reduction in limiting deoxynucleoside triphosphate pools. Under these conditions, increases as well as decreases in deoxynucleoside triphosphate pools would contribute to the inhibition.

Since the overall rate of DNA synthesis appears to be the average rate of incorporation of each deoxynucleotide, a critical balance in all four deoxynucleotides appears required for the optimum rate of DNA synthesis.

[1] Steinberg, J. A., G. B. Grindey. Isolation of a DNA polymerase α associated regulatory protein from calf thymus (submitted).

In conclusion, analysis of perturbations in deoxyribonucleoside triphosphate pools induced by deoxynucleosides in mammalian cells under conditions that inhibit DNA synthesis indicate that the concentrations of these pools are directly related to the rate of DNA synthesis. The results also indicate that small perturbations in the deoxynucleotide pools dramatically inhibit cellular DNA synthesis and that the mechanism of action of many antimetabolites may be related to induced imbalances in the deoxynucleoside triphosphate pools.

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