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Analysis of the drug synergism between thymidine and arabinosyl cytosine using mouse S49 T lymphoma mutants

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Summary. *The synergism between arabinosyl cytosine (araC) and thymidine is characterized using two mutant S49 T lymphoma cell populations with altered deoxyribonucleotide metabolism. AraC-1-6 cells are deficient in dCMP deaminase activity resulting in a secondary elevation of intracellular dCTP pools, whereas dGuo-200-l cells have a mutation in the M_1 subunit of ribonucleotide diphosphate reductase, which also results in elevation of dCTP levels. These two mutant cell populations are partially resistant to araC cytotoxicity as compared to the wild type cells. The resistance to araC is contributed to the elevation of dCTP levels in these mutants which prevent araC incorporation into the DNA due to feedback inhibition of deoxycytidine kinase. Addition of extracellular thymidine to dCMP deaminase deficient cells causes a decrease in dCTP levels and in parallel increase their sensitivity to araC. In contrast, extracellular thymidine does not reduce dCTP levels in the mutant cells with altered ribonucleotide reductase and no synergism between araC and thymidine is observed in these cells. The expansion of dTTP pools in the presence of thymidine is similar in the two mutants. These results suggest that the depletion of dCTP pools by thymidine is responsible for the synergistic action of thymidine on araC cytotoxicity and that dTTP does not directly enhance the incorporation of araC into the DNA of T lymphoma cells.*

Introduction

1-β-D-Arabinofuranosylcytosine (araC) inhibits the growth of various mammalian cell lines and tumors [3, 6, 18] and is used in the treatment of acute myelocytic leukemia [1]. The cytotoxicity of araC is dependent upon its phosphorylation to araCTP and there is a correlation between the formation and retention of araCTP and its efficacy in leukemia treatment [15]. The phosphorylation of araC is catalyzed by deoxycytidine kinase, which is sensitive to feedback inhibition by dCTP [12]. As a result, the phosphorylation of araC and its efficacy as an anticancer agent are related to the level of intracellular dCTP [13, 14, 16]. Therefore, agents that cause a reduction of intracellular dCTP level may have a synergistic effect on araC toxicity.

Thymidine acts synergistically with araC in inhibiting the growth of leukemic cells in vitro and in vivo [2, 7, 11, 18]. Thymidine salvage by leukemic cells causes an expansion of the intracellular dTTP pool, which inhibits CDP reduction resulting in depletion of intracellular dCTP pools [18]. Depletion of the dCTP level by exogenous thymidine may enhance the accumulation of araCTP and thus could conceivably result in an increased cellular sensitivity to growth inhibition by araC [18]. An alternative explanation for the synergism between araC and thymidine has been suggested by Kinahan et al. [11]. According to this explanation, an elevated level of dTTP could enhance the phosphorylation of araC by interfering with the inhibition of deoxycytidine kinase by dCTP [5]. In the present paper we have used mutant mouse T-lymphoma cells with altered deoxyribonucleotide metabolizing enzymes to test these alternative explanations of the synergism between araC and thymidine.

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Materials and methods

Materials. 5,6-[^{3}H]AraC (15 Ci/mmol) was purchased from Moravek Biochemicals Brea, Calif. Thymidine, araC, araCMP, araCTP, dCTP, dATP, dGTP, and dTTP were purchased from the Sigma Chemical Co., St. Louis, Mo. 8-[^{3}H]dATP (17 Ci/mmol), methyl-[^{3}H]dTTP (44 Ci/mmol), and 5-[^{3}H]dCTP (25 Ci/mmol) were purchased from ICN (Irvine, Calif). Poly [d(A,T)], poly [d(G,C)], and *E. coli* DNA polymerase I were purchased from Miles Chemical Co.

Cell culture. Mouse T-lymphoma (S49) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum. The parent purine nucleoside phosphorylase-deficient (NSU-1) cell line and two mutant derivatives, a dCMP deaminase-deficient (araC-6-1) clone and a clone with altered ribonucleotide reductase (dGuo-200-1) are described elsewhere [17]. For cell growth experiments 5×10^4 cells were incubated in microtiter plates in 0.2 ml Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated (30 min, 56° C) horse serum and containing the indicated concentration of araC. Thymidine treatment was performed by preincubating the cells with 10^{-4} *M* thymidine. After 4 h thymidine was washed out and the cells incubated with the appropriate concentrations of araC. A 4-h incubation with 10^{-4} *M* thymidine did not affect the cell cycle distribution and did not cause any inhibition of cell growth. Cells were counted in a Coulter cell counter (Coulter Electronic, Hialea, Fla).

Analysis of [^{3}H] ara-C incorporation. Cells (1×10^6/ml) were incubated for 2 h with [^{3}H]AraC (15 Ci/mmol), and this incubation was preceded, where appropriate, by a 2-h preincubation with thymidine. At the end of the incubation period nucleotides were extracted with 0.4 *M* cold perchloric

acid and neutralized with potassium hydroxide. Nucleotides were separated by thin-layer chromatography on polyethyleneiminecellulose plates according to Crabtree and Henderson [4] in the presence of nonradioactive carriers. Spots corresponding to araCMP, araCDP, and araCTP were visualized under ultraviolet light; they were cut out and the radioactivity counted. In all experiments > 90% of the radioactivity was found in [^{3}H]araCTP. The incorporation of [^{3}H]araC into DNA was analyzed as described by Hunting et al. [10].

Deoxyribonucleoside triphosphate measurements. Nucleotides were extracted as described above. Deoxyribonucleoside triphosphates were assayed by the DNA polymerase method as described by Hunting and Henderson [9]. Poly [d(A,T)] and poly [d(G,C)] were used as templates for the reaction catalyzed by *E. coli* DNA polymerase I.

Results and discussion

Three mutant S49 lymphoma cell populations were used in the present study. NSU-1 cells are the parent purine nucleoside phosphorylase-deficient cells from which two additional mutants were selected as described by Weinberg et al. [17]. The NSU-1 cells have similar dNTP pools to wild-type cells, and for the purpose of these studies are similar to the wild type [17]. AraC-1-6 cells were selected for resistance to 6 μ*M* araC and are deficient in dCMP deaminase activity with secondary increased intracellular dCTP levels [17]. dGuo-200-1 cells were selected for resistance to deoxyguanosine and contain a mutation in the protein M_1 subunit of ribonucleoside diphosphate reductase, resulting in the loss of normal feedback inhibition by dATP and in elevated deoxyribonucleoside triphosphates levels, including dCTP [17]. The sensitivity of the three cell populations to growth inhibition by araC is depicted in Fig. 1. Both the dCMP deaminase-deficient mutant (araC-6-1) and the defective ribonucleoside diphosphate reductase mutant (dGuo-200-1) are resistant to inhibition of growth by higher araC concentrations (50% inhibition at 3.0 μ*M* and 0.8 μ*M* araC, respectively) than in the parent cells (NSU-1) (50% inhibition at 0.2 μ*M* araC).

To test for synergism between thymidine and araC, the cells were pulsed with thymidine (10^{-4} *M*) for 4-h, thymidine was washed out, and the cells were incubated with different concentrations of araC and growth followed for 3 days (Fig. 2). The results clearly show the existence of synergism between thymidine and araC in inhibiting the growth of the parent cells (Fig. 2, panel A) and in the dCMP deaminase-deficient cells (Fig. 2, panel B). In contrast, there is no synergism between thymidine and araC in the mutant with altered ribonucleotide reductase activity (Fig. 2, panel C).

In view of the close correlation between araC toxicity and the degree of araCTP accumulation [13, 14, 16], we have determined the effect of increasing thymidine concentrations on both araCTP accumulation and araC incorporation into DNA (Fig. 3). Indeed, there is good correlation between the degree of synergism of thymidine and araC in the three cell populations (Fig. 2) and its effect on both araCTP accumulation (Fig. 3A) and araC incorporation into the DNA (Fig. 3B). The effect of thymidine on both araCTP accumulation and on araC incorporation into the DNA is more pronounced in the dCMP deaminase-deficient cells (araC-6-1)

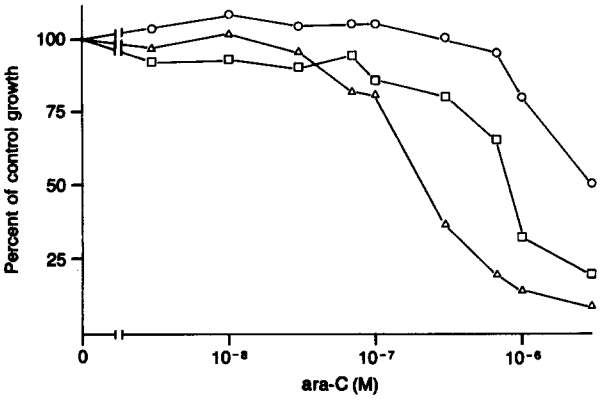

Fig. 1. Growth inhibition of S49 cells by araC. Cells were incubated with the indicated araC concentrations and the number of viable cells counted after 72 h. See *Materials and methods* for additional details. The curves show the numbers of cells of parent cell line (NSU-1, ○---○); cells containing a dATP-resistant ribonucleotide reductase (dGuo-200-1, □---□); and dCMP deaminase-deficient cells (araC-6-1, △---△). The results recorded are means obtained in a single experiment done in triplicate cultures. The range of the triplicate samples did not exceed ± 10% of the mean. Three additional experiments gave similar results

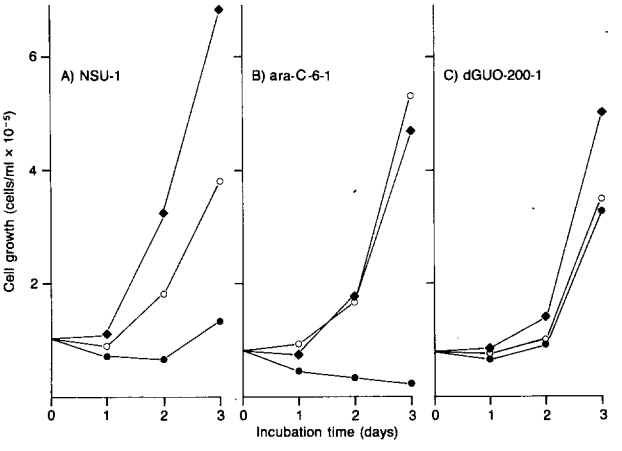

Fig. 2. Synergism between araC and thymidine in growth inhibition of T-lymphoma cell mutants. NSU-1 (*panel A*), araC-6-1 (*panel B*), and dGuo-200-1 (*panel C*) cells were preincubated in the presence or absence of thymidine (0.1 m*M*) for 4-h. The thymidine was washed out, and the cells were incubated, in the presence or absence of araC until harvested and counted. The araC concentrations used were 0.1, 0.2, and 0.7 μ*M* for NSU-1, dGuo-200-1, and araC-6-1 cells, respectively. Additional details are given in *Materials and methods*. The results recorded are means obtained in a single experiment done in triplicate. The range of the triplicate samples did not exceed ± 10% of the mean. Two additional experiments gave similar results. (○---○), 4-h preincubation with thymidine followed by continued incubation in the presence of araC; (●---●), 4-h preincubation without thymidine followed by continued incubation in the presence of araC; (◆---◆), 4-h preincubation with thymidine followed by continued incubation without araC

than in the parent cells (NSU-1); in contrast, thymidine has no significant effect on araC metabolism in the cells with the ribonucleotide reductase mutation (dGuo-200-1).

To determine the ralationship between intracellular dCTP pool sizes and araCTP accumulation, we have determined the intracellular dCTP levels in these cells in response to increasing thymidine concentrations (Fig. 4A) and compared the results with the effect of thymidine on araCTP accumulation from exogenous araC in these cells. Intracellular dCTP levels are the highest in dCMP deaminase cells but are rapidly depleted in the presence of increasing thymidine concentrations. On the other hand, untreated parent cells (NSU-1) have the lowest intracellular dCTP pools, which decrease further in the presence of extracellular thymidine. In contrast, extracellular thymidine does not affect dCTP levels in the cells with the ribonucleotide reductase mutation. Thus, these results demonstrate a good correlation between the effect of thymidine on dCTP levels (Fig. 4A) and the thymidine effects on araCTP accumulation (Fig. 3) and toxicity (Fig. 2) in the three cell populations.

The above results demonstrate the existence of synergism between thymidine and araC in inhibiting the growth of the parental (NSU-1) and dCMP deaminase-deficient cells (ara C-6-1). In contrast, no such synergism exists in the cells with the ribonucleotide reductase mutation (dGuo-100), in which the dCTP pools are unaffected by exogenous thymidine. These data are consistent with the hypothesis that dCTP plays an important role in the synergism between araC and thymidine leading to growth inhibition [2, 7, 8, 11].

To determine whether the increased intracellular dTTP pool affects the phosphorylation of araC independent of its effect on dCTP pools, we measured the levels of dTTP in the presence of increasing thymidine concentrations (Fig. 4B). All three cell populations can effectively accumulate dTTP from thymidine. However, although the cells with a ribonucleotide reductase mutation (dGuo-200-1) can efficiently accumulate intracellular dTTP in the presence of added thymidine (Fig. 4B), thymidine does not affect either araC toxicity (Fig. 2) or araCTP accumulation in these cells (Fig. 3). These results clearly demonstrate that in S49 T-lymphoma cells the synergism between thymidine and araC is based only on the depletion of intracellular dCTP pools and not on the stimulation of deoxycytidine kinase by elevated dTTP pools.

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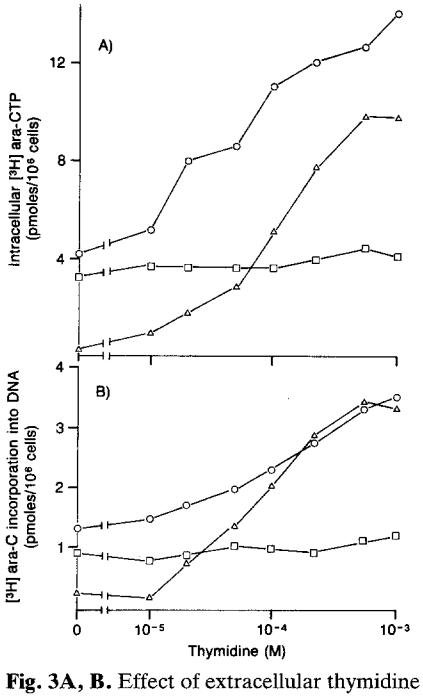

Fig. 3A, B. Effect of extracellular thymidine pretreatment on araCTP accumulation and on araC incorporation into DNA in T-lymphoma mutant cells. Cells were preincubated with the indicated thymidine concentration. After 2-h the thymidine was washed out and [^{3}H]araC (0.5 μCi, 0.03 μ*M*) was added). After an additional 2-h incubation the incorporation of radioactivity into araCTP (**A**) and DNA (**B**)) was determined. See *Materials and methods* for additional details. The *curves* show the numbers of parent cells (NSU-1, ○---○), dCMP deaminase-deficient cells (araC-6-1, △---△), and cells with a ribonucleotide reductase mutation (dGuo-200-1, □---□). The results recorded are means obtained in a single experiment done in triplicate. The range of the triplicate samples did not exceed ± 15% of the mean. Three additional experiments gave similar results

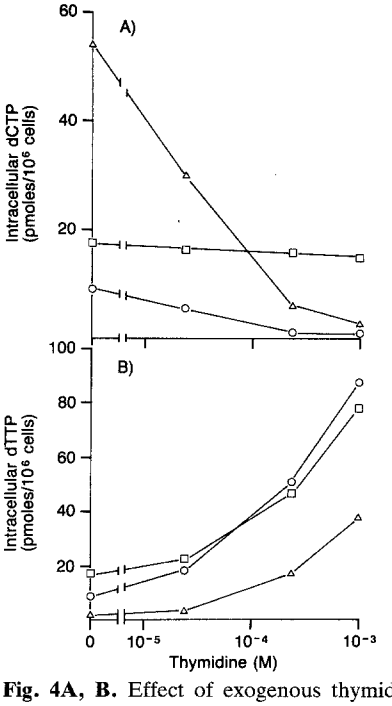

Fig. 4A, B. Effect of exogenous thymidine on intracellular dCTP, dTTP pools in T-lymphoma mutant cells. Cells were incubated with the indicated thymidine concentrations. After 2-h intracellular dCTP (**A**) and dTTP (**B**) levels were determined using the DNA polymerase assay described in details in *Materials and methods*. Numbers of parent cells (NSU-1, ○---○), dCMP deaminase-deficient cells (araC-6-1, △---△), and cells with feedback insensitive ribonucleotide reductase (dGuo-200-1, □---□) are shown. The results recorded are means obtained in a single experiment done in triplicate. The range of the triplicates did not exceed ± 15% of the mean. Three additional experiments gave similar results

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