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THYMIDYLATE SYNTHASE INHIBITION IN CELLS WITH ARRESTED DNA SYNTHESIS IS NOT DUE TO AN ALLOSTERIC INTERACTION IN THE REPLITASE COMPLEX

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SUMMARY: Activity of thymidylate synthase was measured *in situ* in leukemia cells by tritium release from [5-^{3}H]dUrd. Aphidicolin, an inhibitor of DNA polymerase α, but not thymidylate synthase, caused a time dependent inhibition of the enzyme when added to the cells after [5-^{3}H]dUrd. Cells treated with hydroxyurea and aphidicolin in sequence before addition of [5-^{3}H]dUrd had a high initial thymidylate synthase activity that decreased with time. This pattern indicates that thymidylate synthase activity is linked to DNA synthesis; however, its inhibition by drugs that inhibit DNA synthesis may be due to accumulation of thymidine nucleotide(s), rather than to an allosteric interaction in the replitase complex.

Thymidylate synthase (methylenetetrahydrofolate: deoxyuridine-5'-monophosphate C-methyltransferase; EC 2.1.1.45) is the only source of thymidine nucleotides synthesized *de novo* in a cell. We have previously shown that this enzyme activity assayed *in situ* in mouse leukemia L1210 cells by measuring tritium released from [5-^{3}H]dUrd is inhibited when DNA synthesis is arrested in certain phases of the cell cycle or due to inhibition of ribonucleotide reductase by hydroxyurea (1,2). Observations on the time course of thymidylate synthase activity changes following inhibition of DNA synthesis as well as on inhibition of the enzyme activity by exogeneously supplied thymidine allowed us to suggest that the enzyme is regulated by thymidine nucleotide(s) channeled in a multienzyme complex of DNA biosynthesis (2), later named "replitase" (3).

Recently it has been shown that thymidylate synthase activity assayed *in situ* also was cell cycle stage-dependent in Chinese hamster embryo fibroblast cells (4). In these as well as in normal human foreskin

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fibroblast cells thymidylate synthase was inhibited by drugs that caused DNA synthesis arrest (5,6). These results were interpreted by others in terms of allosteric inhibition of thymidylate synthase due to its interaction with other enzymes of the replitase (4-6), rather than by thymidine nucleotides (2).

Evidence is presented here that in mouse (L1210) and human (CCRF-CEM and SKL7) tumor cells with DNA synthesis arrested by a DNA polymerase α inhibitor, aphidicolin (5-7), initial thymidylate synthase activity is similar to that found in the control cells. Thus the enzyme seems to be inhibited by accumulating thymidine nucleotide(s) and not by an allosteric interaction in the replitase complex.

MATERIALS AND METHODS

Cell culture media and sera were obtained from Grand Island Biological Co. [5-^{3}H]dUrd (22 Ci/mmol) was purchased from Moravek Biochemicals and [2-^{14}C]dThd (55 mCi/mmol) from New England Nuclear. Activated charcoal was from J.T. Baker Chemical Co and aphidicolin, hydroxyurea and dUrd from Sigma Chemical Co. All other chemicals were of reagent grade.

Cell Culture: L1210 mouse leukemia cells were grown as described earlier (2). Human leukemia CCRF-CEM and SKL7 cells were maintained in Roswell Park Memorial Institute medium 1640 containing 10% fetal calf serum, 100 units/ml of penicillin (base), and 100 μg/ml of streptomycin (base). The doubling time of the cells was 18 h for both human lines. Exponentially growing cells were used in all experiments.

Assay of Thymidylate Synthase Activity In Situ: The assay was performed as described earlier (2) except that specific radioactivity of [5-^{3}H]dUrd was 5 x 10^{8} cpm/μ mol. All assays were done in duplicate.

Assay of DNA Biosynthesis In Situ: Incorporation of [2-^{14}C]dThd at 37° into the acid insoluble fraction was followed. To a 100 μl sample of cell suspension (about 1.5 x 10^{6} cells/ml), prepared as described earlier (2), [2-^{14}C]dThd was added to a final concentration of 2 μM (L1210 and CCRF-CEM) or 10 μ M (SKL7). To stop incubation 100 μl of ice-cold 10% perchloric acid was added and the sample cooled in ice. The precipitated material was separated by centrifugation (8000 xg, 1 min) and washed three times with 0.5 ml of ice-cold 2% perchloric acid. The final pellet was heated with 0.5 ml of 16% perchloric acid at 80° for 30 min and the solubilized radioactivity determined.

Affinity chromatography on 10-formyl-5,8-dideazafolate-ethyl-Sepharose (8) was used to purify L1210 and CCRF-CEM thymidylate synthase. Activity of the purified enzyme was assayed as described previously (8).

RESULTS AND DISCUSSION

Time courses of tritium release from [5-^{3}H]dUrd (reflecting thymidylate synthase activity) and of thymidine incorporation into acid-insoluble fraction (reflecting DNA synthesis) were studied in exponentially

growing mouse L1210, and human CCRF-CEM (9) and SKL7 (10) leukemic cells exposed to aphidicolin. This DNA polymerase α inhibitor was added either 5 min after [5-^{3}H]dUrd or 10 min after hydroxyurea and 5 min before [5-^{3}H]dUrd was added.

Aphidicolin (2 μg/ml) caused immediate and almost complete inhibition of [^{14}C]thymidine incorporation by all the cell lines studied (Figures 1A, 2A,3A). A similar effect of the drug on DNA synthesis in several other human and murine neoplastic cell lines has been reported (11). When added after [5-^{3}H]dUrd, it also inhibited the release of tritium. However, the latter inhibition (i) always started at least a few minutes after addition of the drug, (ii) was never completed in 40 min, and (iii) was distinctly stronger in L1210 and CCRF-CEM than in SKL7 cells (Figure 1B, 2B, 3B).

In cells pretreated with hydroxyurea and studied in the presence of both hydroxyurea and aphidicolin, the initial rate of the tritium release, observed immediately after addition of [5-^{3}H]dUrd, was comparable to that found in control cells but decreased markedly with time (Figures 1B, 2B, 3B). Neither hydroxyurea (1 mM) nor aphidicolin (2 μg/ml) inhibited the activity of purified L1210 or CCRF-CEM thymidylate synthase preparations. Neither drug affected thymidylate synthase activity in crude extracts from SKL7 cells.

Inhibition by aphidicolin of thymidylate synthase activity in intact cells (Figures 1B, 2B, 3B) and the lack of sensitivity to the drug of the enzyme studied under cell-free conditions (see ref. 5) indicate that thymidylate synthase activity in intact cells is linked with DNA polymerase α activity. However, the linkage does not seem to be very tight, since thymidylate synthase inhibition was always distinctly delayed in relation to DNA synthesis inhibition (Figure 1-3, compare A and B). Such a delay would not be expected if thymidylate synthase was inhibited due to allosteric interaction caused by DNA polymerase α inhibition, as suggested by Reddy and Pardee (5). On the other hand, it should be

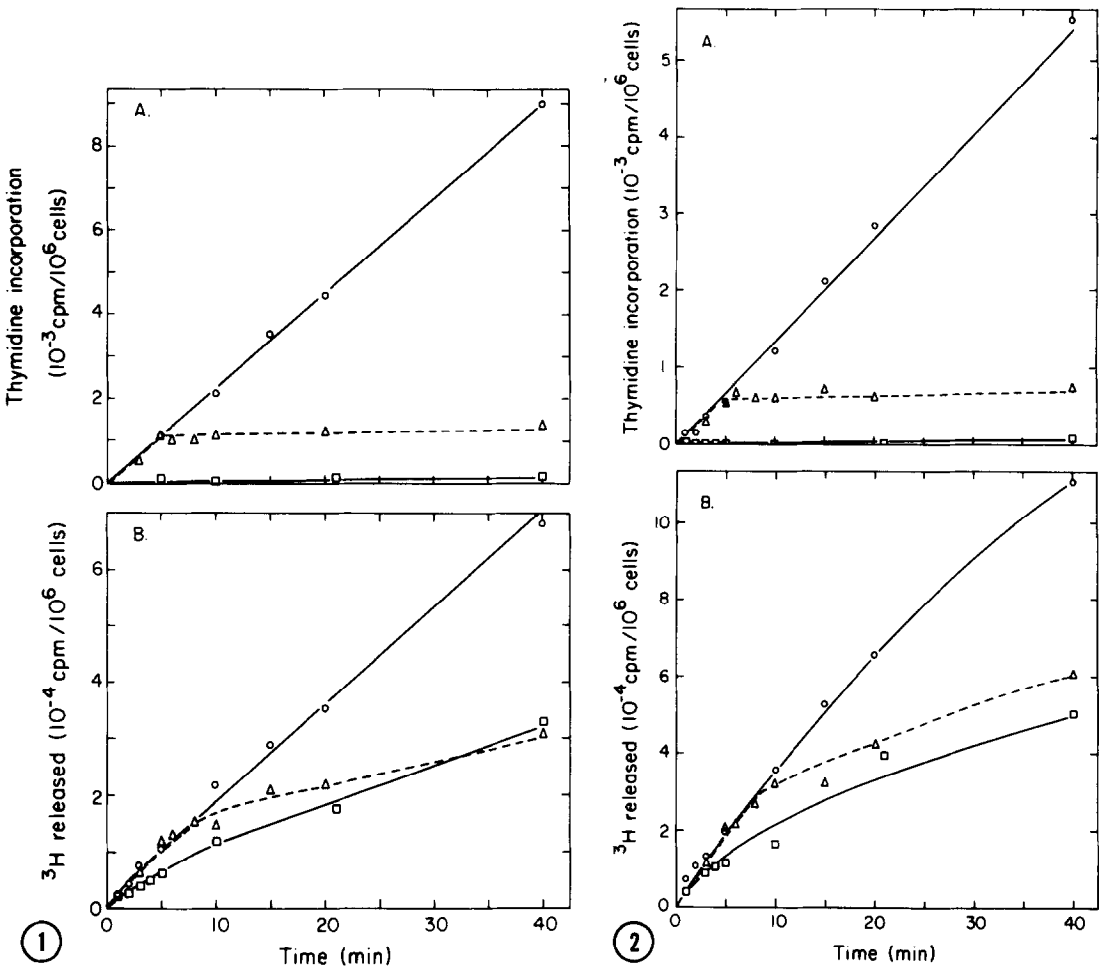

Figure 1. Influence of aphidicolin (2 μg/ml) on (A) thymidine incorporation into acid-soluble material and (B) activity of thymidylate synthase *in situ* in L1210 cells. ○-○ , control without additions; △- -△ , aphidicolin added 5 min after [^{14}C]dThd (A) or [5-^{3}H]dUrd (B); □-□ , [^{14}C]dThd (A) or [5-^{3}H]dUrd (B) added 15 min after hydroxyurea (1 mM) an 5 min after aphidicolin.

Figure 2. Influence of aphidicolin (2 μg/ml) on (A) thymidine incorporation into acid-insoluble material and (B) activity of thymidylate synthase *in situ* in CCRF-CEM cells. All symbols as in legend to Figure 1.

expected if thymidylate synthase inhibition was due to thymidine nucleotide(s) accumulation as we suggested earlier (2).

Of particular interest is the rather weak inhibition of thymidylate synthase activity observed in aphidicolin-treated SKL7 (Figures 3B) as compared with CCRF-CEM (Figure 2B) cells. Since DNA synthesis in both lines showed similar sensitivity to the drug (Figures 2A, 3A), then the

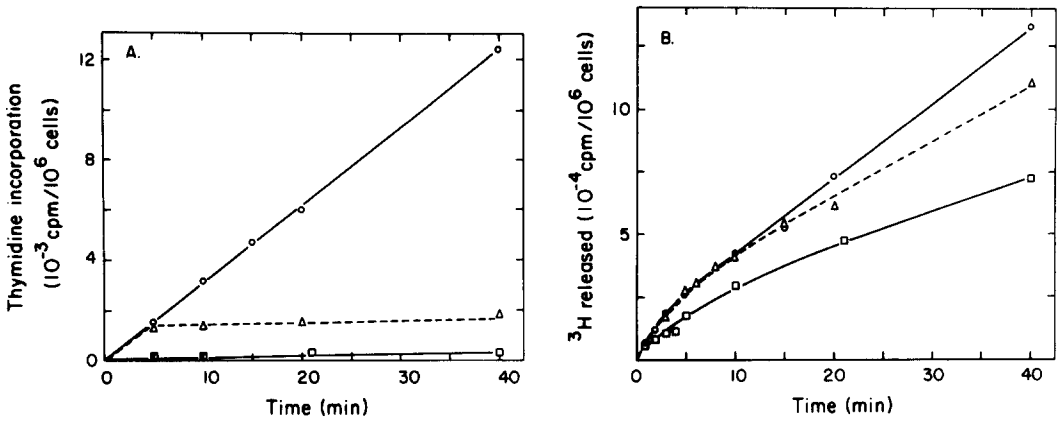

Figure 3. Influence of aphidicolin (2 µg/ml) on (A) thymidine incorporation into acid-insoluble material and (B) activity of thymidylate synthase in situ in SKL7 cells. All symbols as in legend to Figure 1.

linkage between thymidylate synthase and DNA polymerase α activities in SKL7 cells seems to be less tight. A possible explanation of this phenomenon may be related to the different origins of these cell lines. CCRF-CEM cells are of T (12) while SKL7 cells are of B (13) lymphocyte lineage. Human T leukemic lines were found to be much more sensitive to growth inhibition by thymidine than B lines (14-16). For example, results also obtained in our laboratory show different sensitivity of CCRF-CEM and SKL7 cells towards thymidine (ED_{50} = 1.2 x 10^{-5} M and 5 x 10^{-4}), respectively. Thymidine sensitivity was correlated with reduced activity of enzymes catabolizing thymidine and its nucleotides, resulting in the development of a large thymidine triphosphate pool after exposure to thymidine (14,16,17). Thus, rapid catabolism of thymidine nucleotides in the B (SKL7) lymphocytes might be reflected by a less tight link between thymidylate synthase and DNA polymerase α activities if thymidine nucleotides were responsible for the linkage.

The hypothesis that allosteric interaction in the replitase multienzyme complex caused thymidylate synthase inhibition (4-6), was tested by examination of this enzyme activity in cells with DNA synthesis arrested before addition of [5-^{3}H]dUrd. To prevent accumulation of thymidine nucleotides after aphidicolin treatment and before [5-^{3}H]dUrd

addition, cells were pretreated with hydroxyurea, which is known to inhibit ribonucleotide reductase (18-22). Since under conditions of DNA synthesis arrested with aphidicolin (and hydroxyurea) in all cell lines studied initial thymidylate synthase activity was comparable to that found in control cells (Figure 1B, 2B, 3B), we believe the hypothesis that the enzyme is inhibited due to allosteric interaction in the replitase is incorrect. Thymidylate synthase in intact cells seems to be unperturbed by DNA polymerase α inhibition by aphidicolin or ribonucleotide reductase inhibition by hydroxyurea (see our earlier experiments on hydroxyurea-pretreated L1210 cells, ref. 2). Time-dependent inhibition of the enzyme may be due to the addition of deoxyuridine, a precursor of thymidine nucleotides.

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REFERENCES

1. Rode, W., Scanlon, K.J. and Bertino, J.R. (1979) Chemistry and Biology of Pteridines (Kisliuk, R.L. and Brown, G.M., eds.), pp. 489-494, Elsevier North-Holland, New York-Amsterdam-Oxford.
2. Rode, W., Scanlon, K.J., Moroson, B.A. and Bertino, J.R. (1980) J. Biol. Chem. 255, 1305-1311.
3. Reddy, G.P.V. and Pardee, A.B. (1980) Proc. Natl. Acad. Sci. USA 77, 3312-3316.
4. Reddy, G.P.V. (1982) Biochem. Biophys Res. Commun. 109, 908-915.
5. Reddy, G.P.V. and Pardee, A.B. (1983) Nature 304, 86-88.
6. Boorstein, R.J. and Pardee, A.B. (1983) Biochem. Biophys. Res. Commun. 117, 30-36.
7. Huberman, J.A. (1981) Cell 23, 647-648.
8. Rode, W., Scanlon, K.J., Hynes, J. and Bertino, J.R. (1979) J. Biol. Chem. 254, 11538-11543.
9. Foley, G.E., Lazarus, H., Farber, S., Uzman, B.G., Boone, B.A. and McCarthy, R.E. (1965) Cancer 18, 522-529.
10. Clarkson, B., Strife, A. and de Harven, E. (1967) Cancer 20, 926-947.
11. Pedrali-Noy, G., Belvedere, M., Crepaldi, T., Focher, F. and Spadari, S. (1982) Cancer Res. 42, 3810-3813.
12. Kaplan, J., Mastrangelo, R. and Peterson, W.D., Jr. (1974) Cancer Res. 34, 521-525
13. Clarkson, B.D., Fried, J., Chou, T.-C., Strife, A., Ferguson, R., Sullivan, S., Kitahara, T. and Oyama, A. (1977) Cancer Res. 37, 4506-4522.
14. Fox, R.M., Piddington, S.K., Tripp, E.H., Dudman, N.P. and Tattersall, M.H.N. (1979) Lancet II, 391-393.

15. Fox, R.M., Tripp, E.H., Piddington, S.K. and Tattersall, M.H.N. (1980) Cancer Res. 40, 3383-3386.
16. Fox, R.M., Piddington, S.K., Tripp, E.H. and Tattersall, M.H.N. (1981) J. Clin. Invest. 68, 544-552.
17. Carson, D.A., Kaye, J., Matsumoto, S., Seegmiller, J.E. and Thompson, L. (1979) Proc. Natl. Acad. Sci. USA 76, 2430—2433.
18. Young, C.W. and Hodas, S. (1964) Science 146, 1172-1174.
19. Adams, R.L.P, and Lindsay, J.G. (1967) J. Biol. Chem. 242, 1314-1317.
20. Young, C.W., Schochetman, G., and Karnofsky, D.A. (1967) Cancer Res. 27, 526-534.
21. Krakoff, J.H., Brown, N.C. and Reichard, P. (1968) Cancer Res. 28, 1559-1565.
22. Turner, M.K., Abrams, R. and Lieberman, T. (1966) J. Biol. Chem. 241, 5777-5780.