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CORRELATION OF THYMIDINE-ENHANCED INCORPORATION OF ARA-C IN DEOXYRIBONUCLEIC ACID WITH INCREASED CELL KILL*

PIERRE P. MAJOR†, LALLA SARGENT, E. MICHAEL EGAN and DONALD W. KUFE‡
Division of Medicine, Sidney Farber Cancer Institute - Harvard Medical School, Boston, MA 02115, U.S.A.

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Abstract—The treatment of L1210 cells with 10^{-3} or 10^{-4} M thymidine prior to 1-β-D-arabinofuranosylcytosine (ara-C) exposure increased significantly the incorporation of ara-C in DNA. Furthermore, the increased formation of (ara-C)DNA correlated with an enhanced loss of clonogenic survival ($P < 0.0001$). Thymidine pretreatment at either concentration for 16 hr was more effective than a 2-hr pretreatment. The results suggest that the increased formation of (ara-C)DNA is, in part, related to the enhancement of cells traversing through S-phase following thymidine exposure. These results extend our previous observations showing a highly significant relationship between the extent of ara-C incorporation in DNA and loss of clonogenic survival. The incorporation of ara-C in DNA is a useful and easily measurable parameter for evaluating the effects of agents that modulate ara-C metabolism.

We have recently employed cesium sulfate gradient density centrifugation to monitor the incorporation of 1-β-D-arabinofuranosylcytosine (ara-C) into cellular nucleic acids. This approach permits a distinct separation of RNA and DNA under non-degrading experimental conditions. The results obtained with L1210 cells demonstrate specific incorporation of ara-C into DNA with <1 percent of total incorporation into nucleic acid being detectable in RNA [1]. Further, we have found a highly significant relationship ($P < 0.0001$) between the incorporation of ara-C into DNA and the loss of clonogenic survival. Similar results have been obtained using the human HL-60 promyelocytic cell line and blasts from a patient with acute myelogenous leukemia [2]. These results suggested that the formation of (ara-C)DNA was one mechanism responsible for producing lethal cellular events. It was of interest to determine whether the incorporation of ara-C would serve as a reliable biochemical parameter when attempting to enhance the activity of ara-C with modulating agents.

Plagemann *et al.* [3] have shown that various inhibitors of ribonucleotide reductase and of *de novo* pyrimidine biosynthesis enhance the incorporation of ara-C into the nucleotide pool of hepatoma cells. These studies also demonstrated an enhancement by thymidine (dThd) of the incorporation of ara-C into DNA. The effects of dThd on the incorporation of ara-C into DNA could occur as a result of dCTP pool depletion and also by induction of a synchronous progression of cells through S-phase following dThd exposure [4]. Non-cytotoxic concentrations of dThd have been shown to enhance the sensitivity of cells to ara-C and this correlates with the concomitant depression of dCTP pools [5]. Similar observations have been reported in L1210 cells and have been extended by showing that dThd enhances the intracellular and nucleic acid incorporation of ara-C, probably through the reduction in dCTP pools [6].

We have also employed dThd as a means of enhancing ara-C incorporation into cellular DNA to determine whether the increased formation of (ara-C)DNA correlates with changes in clonogenic survival. The results demonstrate that dThd pretreatment enhances the incorporation of ara-C into DNA and that this parameter correlates with increases in cell kill. The increased formation of (ara-C)DNA is, in part, related to the enhancement of cells traversing through S-phase following dThd exposure. The incorporation of ara-C in DNA is a useful and easily measurable parameter for evaluating the effects of modulating agents on ara-C metabolism.

MATERIALS AND METHODS

Cell culture. The L1210 cells were grown as described previously [1].

Thymidine pretreatment. Thymidine (Sigma Chemical Co., St. Louis, MO) was freshly prepared in complete SMEM§ with 10% heat-inactivated, dialyzed fetal calf serum at a concentration of 10^{-2} M. The stock solution was sterilized by Millipore filtration, and serial dilutions were made to give final concentrations of 10^{-3} and 10^{-4} M. Cells were exposed to dThd (10^{-3} or 10^{-4} M) for either 2 or 16 hr, washed, and then monitored for the incor-

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† Fellow of the Medical Research Council, Canada.

‡ Address reprint requests to: Donald W. Kufe, M.D., Division of Medicine, Sidney Farber Cancer Institute, 44 Binney St., Boston, MA 02115, U.S.A..

§ Minimum essential medium (Eagle's) for suspension cultures.

poration of ara-C into DNA and loss of clonogenic survival after ara-C exposure [1].

[^{3}H]*Ara-C incorporation studies.* L1210 cells in logarithmic growth phase were washed twice with phosphate buffered saline (PBS) and were resuspended at 5×10^5 cells/ml in SMEM. The cells were incubated with 13 μCi/ml [^{3}H]ara-C (26 Ci/mmole; New England Nuclear Corp., Boston, MA) and 7.5 μCi/ml [^{32}P]H_2PO_4 (carrier-free; New England Nuclear Corp.) for varying time periods (0.5 to 6.0 hr). The cells were then washed twice with 5 ml PBS, resuspended in PBS at 1×10^7 cells/ml, and digested by the addition of 2.5 mg of pronase B (Calbiochem-Behring Corp., La Jolla, CA) in 2 ml of 0.01 M Tris, (pH 7.4), 0.01 M EDTA and 0.5% sodium dodecylsulfate (SDS). Further purification was accomplished by chloroform–isoamyl alcohol (24:1) extraction. The nucleic acids were precipitated by the addition of 0.4 M NaCl and 2 vol. of absolute ethanol. The samples were then analyzed by $CsSO_4$ centrifugation [1].

RESULTS

Figure 1 shows a representative profile of the incorporation of [^{3}H]ara-C and ^{32}P into RNA and DNA of L1210 cells following dThd pretreatment. Significant amounts of [^{3}H]ara-C were detected within the DNA region of the gradient, while there was no significant tritium radioactivity associated with the RNA region. The labeling with ^{32}P provided a measure of the relative rates of nucleic acid synthesis and demonstrated the ability to clearly distinguish RNA from DNA. The tritium detectable in DNA was specific for the incorporation of [^{3}H]ara-C as determined by digestion of the DNA fraction to nucleosides and analysis by high pressure liquid chromatography [1].

The effect of dThd pretreatment on the incorporation of [^{3}H]ara-C into L1210 DNA is illustrated in

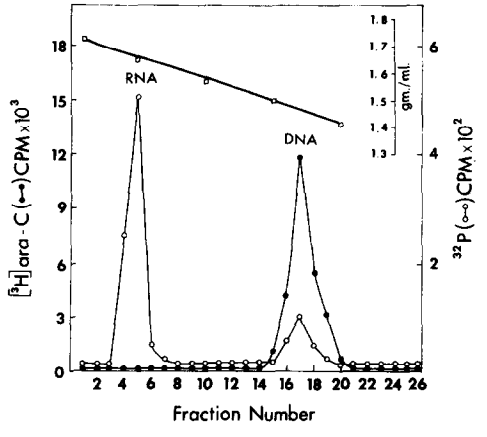

Fig. 1. Incorporation of [^{3}H]ara-C into DNA following thymidine exposure. L1210 cells at 5×10^5/ml were exposed to 10^{-3} M thymidine for 16 hr, washed, and incubated with [^{3}H]ara-C and [^{32}P]H_2PO_4 for 6 hr as described in the text. The nucleic acids from 5×10^6 cells were extracted as described and analyzed by cesium sulfate gradient centrifugation [1].

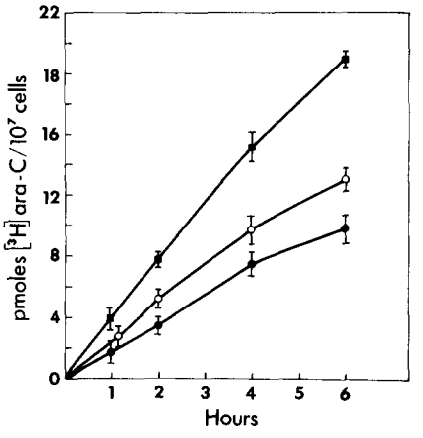

Fig. 2. Effect of dThd pretreatment on [^{3}H]ara-C incorporation into L1210 DNA. L1210 cells at 5×10^5/ml received either no thymidine pretreatment (●) or were pretreated with 10^{-3} M thymidine for 2 (○) or 16 hr (■), and then washed and incubated with 10^{-7} M [^{3}H]ara-C for 1, 2, 4, and 6 hr. The nucleic acids were extracted as described in the text and analyzed by cesium sulfate gradients. The [^{3}H]ara-C counts in the DNA region of the gradient are expressed per 10^7 cells.

Fig. 2. The incorporation of [^{3}H]ara-C in control cells not exposed to dThd was linear during the first 4 hr. The pretreatment of cells with dThd for 2 hr resulted in a significant enhancement of ara-C incorporation into DNA, while 16 hr of dThd pretreatment resulted in even greater increases to levels about 2-fold those observed with the control. Comparable results were obtained with 10^{-4} M dThd pretreatment.

The biologic relevance of enhancing ara-C incorporation into DNA by dThd pretreatment was studied by comparing the amount of ara-C incorporation with the clonogenic potential of the cells after drug exposure. Figure 3A illustrates the effects of dThd pretreatment (2 hr) on the clonogenic survival of cells exposed for 0.5 hr to concentrations of ara-C ranging from 10^{-8} M to 10^{-3} M. This combination resulted in little, if any, enhancement of cell kill. The dotted lines represent the percent of control survival for similar cells in dThd suicide experiments which provide a measure of cells traversing through S-phase during the period of drug exposure [7]. In contrast, a significant enhancement of cell kill was achieved by pretreating cells with dThd for 16 hr, as illustrated in Fig. 3B. Under these conditions, 10^{-3} and 10^{-4} M dThd pretreatment resulted in significant increases in loss of clonogenic survival and greater numbers of cells traversing S-phase during the 0.5 hr ara-C exposure.

The dependence of loss of clonogenic survival on the period of dThd pretreatment should apply also during longer periods of ara-C exposure. Figure 4 illustrates the results achieved with dThd pretreatment for 2 hr (A) and 16 hr (B), followed by ara-C exposure for 3 hr. The dThd pretreatment for 2 hr resulted again in a slight enhancement of cell kill at the higher concentrations of ara-C. In contrast, dThd pretreatment for 16 hr resulted in a nearly 10-fold enhancement in cell kill compared to control. The

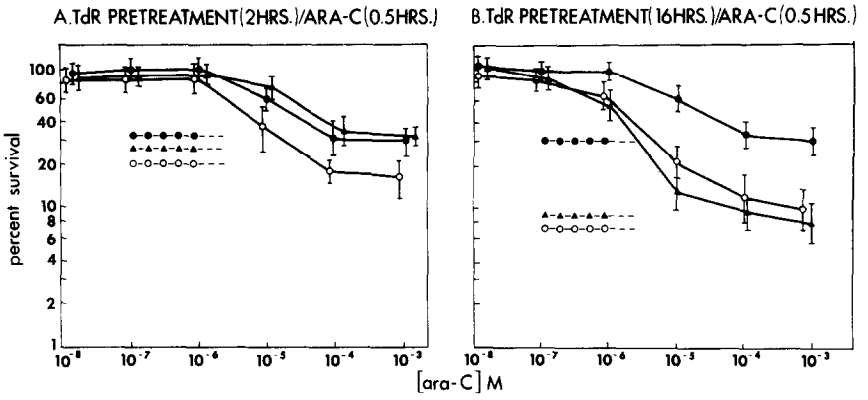

Fig. 3. Effect of dThd pretreatment for 2 hr (A) and 16 hr (B) on clonogenic survival of cells exposed to ara-C for 0.5 hr. L1210 cells at 5×10^5/ml received either no thymidine pretreatment (●) or were pretreated with 10^{-4} M (▲) or 10^{-3} M (○) thymidine. The cells were then washed and exposed to ara-C concentrations of 10^{-8} M to 10^{-3} M. The horizontal lines indicate the percent survival in thymidine suicide experiments under conditions of no thymidine pretreatment (●), pretreatment with 10^{-4} M (▲), or 10^{-3} M (○) thymidine.

increase in loss of clonogenic survival was also accompanied by a concomitant increase in S-phase cells as determined by thymidine suicide experiments.

The relationship between the enhanced incorporation of ara-C into L1210 DNA and the effect of the drug combination on clonogenic survival was evaluated by measuring the amount of [^{3}H]ara-C incorporation into DNA as a result of dThd pretreatment for 2 and 16 hr. Incorporation studies were performed with [^{3}H]ara-C concentrations ranging from 10^{-7} M to 10^{-4} M, and the amounts incorporated into DNA were determined for each ara-C incubation period (0.5 hr and 3 hr). The clonogenic survival at each ara-C concentration was derived from the cloning data shown in Figs. 3 and 4. The correlation of the log pmoles of ara-C incorporated into DNA with the log percent survival was significant for ara-C exposures of both 0.5 hr (Fig. 5A: coefficient $[R] = -0.883$, $P < 0.0001$) and 3 hr (Fig. 5B: coefficient $[R] = -0.866$, $P < 0.0001$). Thus, using a logistic model [8], an enhanced incorporation of ara-C into DNA was significantly associated with a reduced percentage of survival that was similar for ara-C exposures of 0.5 hr and 3 hr. Cells pretreated with dThd for either 2 or 16 hr showed this same relationship, with enhancement of ara-C incorporation into DNA being associated in a predictable fashion with increased cell kill.

DISCUSSION

Previous studies have demonstrated that dThd enhances the incorporation of ara-C in nucleic acids [3, 5, 6]. Although dThd enhanced cell kill, no attempt was made to relate this parameter to formation of (ara-C)DNA. Our results demonstrated increased incorporation of ara-C specifically in DNA of L1210 cells pretreated with dThd. Furthermore, there was a highly significant relationship between the incorporation of ara-C in DNA of cells pretreated with dThd and enhanced loss of clonogenic survival.

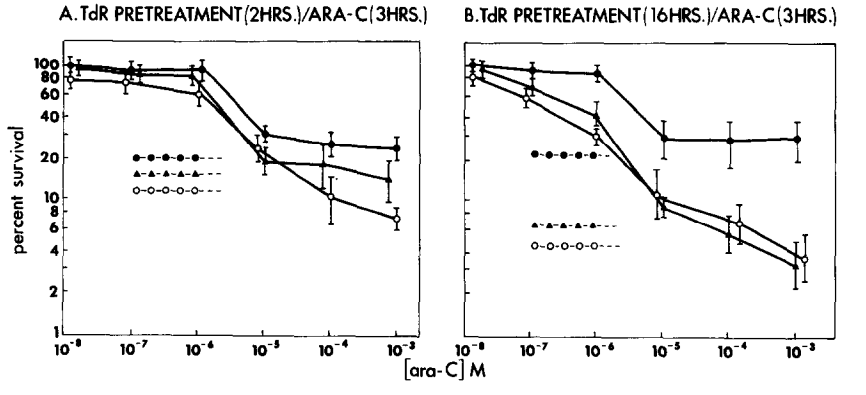

Fig. 4. Effect of dThd pretreatment for 2 hr (A) and 16 hr (B) on clonogenic survival of cells exposed to ara-C for 3 hr. L1210 cells at 5×10^5/ml received either no pretreatment with thymidine (●) or were pretreated with 10^{-4} M (▲) or 10^{-3} M (○). The cells were then washed and exposed to ara-C at concentrations of 10^{-8} M to 10^{-3} M. The horizontal lines indicate the percent survival in thymidine suicide experiments under conditions of no thymidine pretreatment (●) or pretreatment with 10^{-4} M (▲) or 10^{-3} M (○) thymidine.

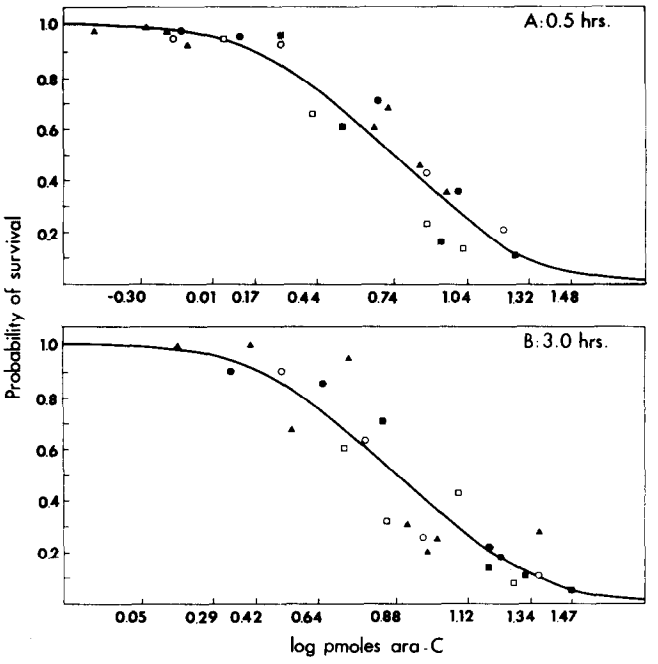

Fig. 5. Probit analysis of the relationship between incorporation of ara-C in DNA and loss of L1210 clonogenic survival at ara-C concentrations of 10^{-7} to 10^{-4} M for 0.5 hr (A) and 3 hr (B). Cells received either no thymidine pretreatment (▲), pretreatment with 10^{-4} M thymidine for 2 (●) or 16 (■) hr, or pretreatment with 10^{-3} M for 2 (○) or 16 (□) hr.

Cells pretreated with dThd for 16 hr formed more (ara-C)DNA than cells treated for 2 hr. Further, there was a corresponding increase in cytotoxicity with the 16-hr pretreatment.

Thymidine exposure has been shown to decrease dCTP pools and to result in slowing of DNA replication, with accumulation of cells in early S-phase. Our studies are consistent with the finding of an increased number of cells progressing through S-phase following dThd exposure. The extent of dCTP pool recovery after dThd treatment, required for the resumption of cell progression through S-phase, remains unclear. Each of these factors may contribute to the enhancement of ara-C incorporation in DNA.

Our observations suggest that the previously established relationship between incorporation of ara-C in DNA and cytotoxicity is maintained when an agent such as dThd is employed to modulate the metabolism of ara-C. These methods are applicable to clinical samples of leukemic blood cells. It has been shown previously that ara-C incorporation in DNA is related to cytotoxicity of human leukemic blasts [2]. It should, therefore, be possible to apply this methodology clinically to monitor the incorporation of ara-C in leukemic cells prior to and after modulation with dThd.

REFERENCES

1. D. W. Kufe, P. P. Major, E. M. Egan and G. P. Beardsley, *J. biol. Chem.* **255**, 8997 (1980).
2. P. P. Major, E. M. Egan, G. P. Beardsley, M. Minden and D. W. Kufe, *Proc. natn. Acad. Sci. U.S.A.*, (in press).
3. P. Plagemann, R. Marz, and R. Wohlhueter, *Cancer Res.* **38**, 978 (1978).
4. G. P. Studzinski and W. C. Lambert, *J. cell. Physiol.* **73**, 109 (1969).
5. A. W. Harris, E. C. Reynolds and L. B. Finch, *Cancer Res.* **39**, 538 (1979).
6. S. Grant, C. Lehman and E. Cadman, *Cancer Res.* **40**, 1525 (1980).
7. A. Becker, E. McCulloch, L. Siminovitch and J. Till, *Blood* **26**, 296 (1965).
8. D. Cox, *Analysis of Binary Data*, pp. 76–99. Barnes & Noble, New York (1969).