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Increased Thymidylate Synthetase Activity in 5-Fluorodeoxyuridine-Resistant Novikoff Hepatoma Cells[1] (39673)

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The fluorinated pyrimidines, including 5-fluorodeoxyuridine (FdUrd), produce objective responses against a variety of human (1) and experimental (2) neoplasms. However, the development of clinical resistance is a major limitation of these drugs (3). Resistance to FdUrd can be induced in Novikoff hepatoma cells by continued exposure to minimally toxic concentrations of the analog (4). The persistence of FdUrd resistance, even after the cells had been grown for at least five generations in the absence of the drug, established the genetic basis for this phenotypic change. The development of FdUrd resistance was accompanied by the loss of thymidine kinase activity (4), the enzyme which is necessary for the conversion of FdUrd to its active metabolite, 5-fluorodeoxyuridylate (FdUMP) (5).

Several enzymes, including thymidine kinase (6), thymidylate synthetase (7), deoxycytidylate deaminase (7), and ribonucleotide reductase (8), seem to be regulated in concert according to the cell's requirements for DNA synthesis. The interrelationships of these enzymes are shown in Fig. 1. In order to see if the deletion of one of these coordinately controlled enzymes would affect the levels of the others, we compared the levels of these enzymes in FdUrd-sensitive (thymidine kinase-containing) and FdUrd-resistant (thymidine kinase-deficient) Novikoff hepatoma cells.

Materials and methods. Cells and media. The Novikoff hepatoma was originally induced as a solid tumor in rats fed 4-dimethylaminoazobenzene (9). The transplantable tumor was subsequently grown in the ascitic form, from which the tissue cultures lines, N1-S1 (thymidine kinase-containing, FdUrd sensitive) and N1-S1/FdUrd (thymidine kinase-deficient, FdUrd resistant), were developed by Morse and Potter (4). Our stock cultures of N1-S1 and N1-S1/FdUrd cells were obtained from Dr. Potter's laboratory (University of Wisconsin, Madison, Wis.) in January 1975. The cells were maintained in suspension culture as described previously (10). In our laboratory, 1×10^{-5} *M* FdUrd completely suppresses the proliferation of N1-S1 cells, but had no effect on the proliferation of N1-S1/FdUrd cells. In fact, the N1-S1/FdUrd cells were routinely carried in medium containing 1×10^{-5} *M* FdUrd to ensure stability of the FdUrd-resistant phenotype. However, for all experiments the N1-S1/FdUrd cells were washed free of FdUrd and cultured in analog-free medium for five generations.

For preparation of cell-free extracts, cells were grown to mid- or late-log phase density and harvested by centrifugation (4080*g*, 10 min, 4°). The cell pellets were washed twice in phosphate buffered-saline (11), and then suspended in 0.02 *M* Tris-HCl buffer, pH 7, containing 1 m*M* dithioerythritol (70×10^7 cells/ml). After allowing the cells to swell for 5 min on ice, the suspension was homogenized by a motor driven homogenizer. The homogenate was centrifuged at 30,000*g* for 1 hr. The supernatant fluid was used as the cell-free enzyme extract.

Thymidine kinase activity was determined by the method of Breitman (12); thymidylate synthetase activity was determined by the radiochemical method of Roberts (13); deoxycytidylate deaminase activity was determined by the method of Maley and Maley using [^{3}H]dCMP as the substrate (14); ribonucleotide reductase activity was determined by the method of Steeper and Steuart (15); ($Na^+ + K^+$)-ATPase activity was determined by the method of Solomonson *et al.* (16); and 5′-nucleotidase activity was determined by the method of Heppel and Hilmoe (17). Protein determinations were made by the method of Lowry *et al.* (18).

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Swim's medium 77 and calf serum were purchased from Grand Island Biological Company (Grand Island, N.Y.) and International Scientific Industries, Inc. (Cary, Ill.), respectively. Pluronic F-68 was a gift from Wyandotte Chemical Company (Wyandotte, Mich.). The nucleosides and nucleotides used in these studies were purchased from Sigma Chemical Co. The ^{14}C- and ^{3}H-labeled compounds were purchased from New England Nuclear and Amersham-Searle.

Results. The levels of the enzymes, thymidine kinase, thymidylate synthetase, deoxycytidylate deaminase and ribonucleotide reductase in N1-S1 and N1-S1/FdUrd cells are given in Table I. The deficiency of thymidine kinase activity seen in the N1-S1/FdUrd cells is similar to that reported by others (19). There was little difference between N1-S1 cells and N1-S1/FdUrd cells in the levels of deoxycytidylate deaminase or ribonucleotide reductase. However, there was a significant increase in the level of thymidylate synthetase activity in the N1-S1/FdUrd cells.

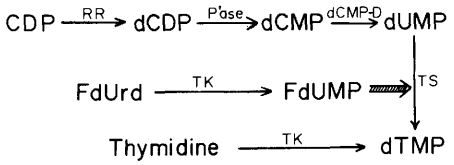

FIG. 1. Pathways for interrelating enzymes involved in deoxyribonucleotide metabolism. The abbreviations used are as follows: ribonucleotide reductase, RR; deoxycytidylate deaminase, dCMP-D; thymidylate synthetase, TS; thymidine kinase, TK; and phosphatase, P'ase. The heavy arrow (⟹) indicates inhibition of thymidylate synthetase by FdUMP.

The sensitivities of the thymidylate synthetase from the N1-S1 and N1-S1/FdUrd tumor cells to inhibition by fluorodeoxyuridylate (F-dUMP) were the same. This was determined at various concentrations of F-dUMP, under conditions of equal protein concentrations and under conditions in which the enzyme activities were equal. At equal protein concentrations, F-dUMP, at a final concentration of 5×10^{-8} *M* inhibited the thymidylate synthetase activity in both the N1-S1 and N1-S1/FdUrd cell-free extracts greater than 98%. The cell-free extract from the N1-S1/FdUrd cells was diluted with a solution of 1% albumin to contain approximately the same amount of enzyme activity per unit volume as the cell-free extract from the N1-S1 cells. Under these conditions, FdUMP (1×10^{-8} *M*) inhibited the thymidylate synthetase activity in the N1-S1 and N1-S1/FdUrd cells by 89 and 87%, respectively.

Although its relationship to DNA synthesis is less direct than that of the enzymes listed in Table I, $(Na^+ + K^+)$-ATPase activity appears to be required for the increased DNA synthetic rate which characterizes the blastogenic response of lymphocytes following phytohemagglutinin stimulation (20). The levels of this enzyme in the N1-S1/FdUrd cells are given in Table II. Also shown are the levels of 5'-nucleotidase, which is a plasma membrane-associated enzyme with no known relationship to DNA synthesis. The specific activity of $(Na^+ + K^+)$-ATPase in N1-S1/FdUrd cells was only one-fifth that present in N1-S1 cells, whereas there was little difference in the 5'-nucleotidase activity.

TABLE I. ENZYME LEVELS IN CELL-FREE EXTRACTS FROM N1-S1 AND N1-S1/FdUrd CELLS.

Experiment	Cell density[b] ($\times 10^{-6}$ cells/ml)	Enzyme activity[a] TK	dCMP-D	TS	RR
		(nmoles/30 min /mg protein)			
I					
N1-S1	1.14	16.0	125	1.99	0.150
N1-S1/FdUrd	0.84	0.17 (0.01)[c]	174 (1.39)	9.47 (4.76)	0.185 (1.23)
II					
N1-S1	0.50	23.0	186	4.73	0.125
N1-S1/FdUrd	0.70	0.15 (0.006)	224 (1.20)	11.11 (2.35)	0.132 (1.06)

[a] The abbreviations used are: TK, thymidine kinase; dCMP-D, deaminase; TS, thymidylate synthetase; RR, ribonucleotide reductase.

[b] Cell density of cultures from which enzyme extracts were prepared.

[c] The numbers in parentheses are the ratio of enzyme levels in the F-dUrd resistant cells to the levels in the F-Urd sensitive cells.

TABLE II. LEVELS OF $(Na^+ + K^+)$-ATPase AND 5'-NUCLEOTIDASE IN THE PLASMA MEMBRANES OF N1-S1 AND N1-S1/FdUrd CELLS.

Experiment	Cell density[a] ($\times 10^{-6}$ cells/ml)	Enzyme Activity ATPase (nmoles of Pi/hr/mg protein)	Enzyme Activity 5'-Nucleotidase (nmoles of Pi/hr/mg protein)
I			
N1-S1	1.14	2.2	–
N1-S1/FdUrd	0.84	0.39 (0.17)[b]	–
II			
N1-S1	0.50	1.03	0.38
N1-S1/FdUrd	0.70	0.20 (0.19)	0.46 (1.21)

[a] The cell density of cultures from which the membrane fraction was prepared (30,000*g* sediment).
[b] The numbers in parentheses are the ratio of the enzyme levels in the resistant cells to the levels in the sensitive cells.

Discussion. The data in Table I show that thymidine kinase activity can be greatly suppressed without causing a concomitant decrease in other enzymes which are usually coordinately regulated with thymidine kinase. This suggests that the mutation which is responsible for the decreased thymidine kinase activity resides in the structural gene for thymidine kinase rather than in a regulatory gene.

The elevated thymidylate synthetase activity in the N1-S1/FdUrd cells may be an adaptive response which allows the *de novo* pathway of dTMP biosynthesis to compensate for decreased dTMP production via the salvage pathway in the thymidine kinase-deficient cells. Alternatively, the elevated levels of thymidylate synthetase present in the N1-S1/FdUrd cells may be an essential part of the FdUrd resistance. The inhibition of thymidylate synthetase activity by FdUMP in cell-free extracts from both N1-S1 and N1-S1/FdUrd cells was similar, indicating that the resistance of N1-S1/FdUrd cells was not due to an altered enzyme which was insensitive to FdUMP. However, the absolute level of residual thymidylate synthetase activity remaining in the resistant cell line after drug therapy (F-uracil or F-deoxyuridine) may be sufficient to allow for nearly normal growth rates. Increased thymidylate synthetase activity has also been reported in cells which are resistant to methotrexate (21), another drug whose primary intracellular target appears to be in the formation of thymidylate.

The significance of the decreased level of $(Na^+ + K^+)$-ATPase activity in the N1-S1/FdUrd cells is unknown. Since the levels of 5'-nucleotidase activity are not significantly different in the two cell lines, the decreased levels of $(Na^+ + K^+)$-ATPase in the N1-S1/FdUrd cells do not appear to be the result of a generalized loss of membrane-associated enzymes. It may, therefore, represent a rather specific alteration in plasma membrane function, possibly affecting nucleoside transport in some manner that decreased the potency of the nucleoside analog. Although rigorous experiments capable of differentiating transport *per se* from uptake have not yet been carried out, preliminary studies show that the N1-S1/FdUrd cells consistently incorporate radioactive ribo- and deoxyribonucleosides into both the acid-soluble and acid-insoluble fractions at a significantly lower level than that observed in the N1-S1 cells.

The multiple enzymatic differences which exist between the N1-S1 and N1-S1/FdUrd cell lines illustrate that the development of drug resistance may be a complex process, involving more than one enzyme locus. These results also emphasize the need to exercise caution when utilizing known genotypic differences between closely related cell lines as an experimental tool, since it is quite likely that unknown differences may easily lead to incorrect conclusions.

Summary. A fluorodeoxyuridine-resistant Novikoff hepatoma cell line, which had been previously characterized as being thymidine kinase deficient, was shown to have elevated levels of thymidylate synthetase. The thymidylate synthetase activity in the fluorodeoxyuridine-resistant cell line was

sensitive to inhibition by FdUMP to the same extent as the fluorodeoxyuridine-sensitive cell line.

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