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STUDIES ON THE MECHANISM OF THE AZASERINE-INDUCED DECREASE IN THE ACTIVITY OF THYMIDINE KINASE*

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Abstract—The intraperitoneal injection of azaserine (*O*-diazoacetyl-L-serine), a potent inhibitor of the synthesis of purine nucleotides *de novo*, into mice bearing sarcoma 180 ascites cells resulted in a decrease to about 25 per cent of normal in the activity of thymidine kinase of the neoplastic cells. Almost complete restoration of the activity of this enzyme was achieved by exposure *in vivo* of the azaserine-treated cells to a source of either preformed adenine or a mixture of thymidine and deoxycytidine. Treatment with azaserine caused a pronounced depletion of adenine nucleotide pools due to an inability of the drug-treated cells to synthesize purine nucleotides *de novo*. Provision to the cells of preformed adenine allowed circumvention of the metabolic blockade and adenine nucleotide pools were restored to normal, whereas the mixture of thymidine and deoxycytidine did not influence the size of the intracellular purine nucleotide pool in cells exposed to azaserine. The results are interpreted and discussed in terms of the role of the substrates in maintaining the intracellular concentration of thymidine kinase.

AZASERINE (*O*-diazoacetyl-L-serine), a cytostatic antimetabolite of glutamine which is produced by a *Streptomyces*, has the capability of inhibiting several glutamine-requiring enzymes. The most sensitive of these enzymes, phosphoribosylformylglycineamidine synthetase [5′-phosphoribosyl-formylglycineamide: L-glutamine amido-ligase (ADP); EC 6.3.5.3], which is irreversibly titrated by azaserine, catalyzes a reaction in the biosynthetic pathway by which purine nucleotides are fabricated *de novo*; thus, essentially complete and prolonged blockade of the formation of purine nucleotides occurs in cells exposed to this agent.[1–15] As a consequence of the inability of azaserine-treated cells to synthesize purine nucleotides, the intracellular pools of adenine and guanine nucleotides are markedly reduced.[15, 16] This phenomenon is accompanied by a pronounced depression in the cellular levels of thymidine kinase (ATP:thymidine 5′-phosphotransferase; EC 2.7.1.21) and thymidine monophosphate kinase (ATP:thymidine monosphosphate phosphotransferase; EC 2.7.4.9). That the decrease in the activities of the thymine nucleotide-synthesizing enzymes in cells exposed to azaserine is associated with the lowered intracellular pools of adenine nucleotides was suggested by the finding that the availability to the cells of a supply of

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preformed adenine restored both the azaserine-depleted pool of adenine nucleotides and the activities of the thymine nucleotide-forming enzymes.[16]

The present report is concerned with the biochemical mechanisms involved in the azaserine-induced depression of the activity of thymidine kinase and the role of adenine nucleotides in regulating the formation of thymine nucleotides, a process of potential importance to the control of the synthesis of DNA.

MATERIALS AND METHODS

Experiments were performed on 9- to 11-week-old female CD-1 mice (Charles River Breeding Laboratories, North Wilmington, Mass., U.S.A.). The animals were inoculated i.p. 6 days before use with 4×10^6 sarcoma 180 ascites cells.[17] The chemical agents were dissolved in isotonic saline just prior to use and were injected i.p.

Preparation of cell-free extracts. Sarcoma 180 ascites cells were collected through an incision in the peritoneal wall; all subsequent procedures were performed at 4°. Cells were treated for 3 min with 4 vol. of 0·2% sodium chloride to lyse erythrocytes; subsequently, an equal volume of 1·6% sodium chloride was added and the cells were collected by centrifugation at 1000 *g*. After two washes with 0·9% sodium chloride, the cells were suspended in 4 vol. of 0·05 M Tris-Cl buffer (pH 7·5) containing 0·05 mM thymidine. The cell suspension was disrupted with a Branson sonifier at 8 amp by one 30-sec burst; tubes containing the cell suspension were immersed in a methanol–ice bath during sonication. The sonicates were centrifuged at 105,000 *g* for 1 hr, and the supernatant fraction was collected. The protein content of the extracts was determined by the biuret method[18] with twice recrystallized bovine albumin as the standard.

Assay for thymidine kinase activity. Thymidine kinase activity was measured by incubating the following mixture at 37°: thymidine-^{3}H, 0·1 μmole ($2{\cdot}9 \times 10^5$ cpm); adenosine triphosphate, 5·5 μmoles; magnesium chloride, 5·5 μmoles; Tris-Cl (pH 7·5), 100 μmoles; and enzyme extract (0·25 ml containing approximately 2 mg protein); total volume, 1·3 ml. The mixture was incubated at 37° for 30 min; throughout this period the enzymatic reaction was linear. The reaction was terminated by the addition of 0·13 ml of 40% perchloric acid, precipitated protein was removed by centrifugation, and the collected supernatant solutions were neutralized to pH 7 with 1 N KOH. After removal of precipitated potassium perchlorate by centrifugation, unreacted thymidine was separated from thymine nucleotides by chromatography on $1{\cdot}3 \times 9{\cdot}5$ cm columns of Ecteola-cellulose (exchange capacity, approximately 0·5 m-equiv./g). Thymidine was eluted with water (70 ml) and the thymine nucleotides with 0·5 N HCl (50 ml). The concentration of thymidine and thymine nucleotides in these fractions was determined by measurement of the radioactivity therein with a Packard Tri-Carb liquid scintillation spectrometer using as the phosphor solution 100 mg of *p-bis*[2-(5-phenyloxazolyl)]benzene and 8 g of 2,5-diphenyloxazole dissolved in 2 l. of toluene and 1 l. of absolute ethanol.

Measurement of adenine nucleotide pool. The size of the acid-soluble adenine nucleotide pool was estimated by isotope dilution;[19] adenine-8-^{14}C HCl hemihydrate (2·80 mμmoles, $2{\cdot}7 \times 10^3$ cpm/mμmole) was added to cold 4% perchloric acid extracts of sarcoma 180 ascites cells, purines were liberated by hydrolysis for 1 hr at 90°, and adenine was purified and its specific activity determined as described earlier.[20]

Chromatography of unhydrolyzed extracts on columns of Dowex 1-formate indicated that essentially all of the isolated adenine was present in nucleotide form.

RESULTS

A pronounced decrease in the activity of thymidine kinase of sarcoma 180 ascites cells was induced by exposure of the tumor cells to azaserine *in vivo*; the decreased activity did not represent direct inhibition of the enzyme by the antibiotic.[16] Furthermore, the availability to the cells of a supply of preformed adenine restored (1) the depleted pool of adenine nucleotides caused by the marked inhibition of the synthesis of purine nucleotides *de novo* by azaserine, and (2) the activity of thymidine kinase. To determine the rate of recovery of thymidine kinase activity after adenine-induced rescue of enzymatic activity, tumor-bearing mice were injected i.p. with 0·2 mg azaserine per kg. Adenine was administered by i.p. injection 11·5 hr after azaserine, and thymidine kinase activity was measured at intervals thereafter. The results shown in Fig. 1 indicate that the cellular activity of thymidine kinase at 12–13 hr

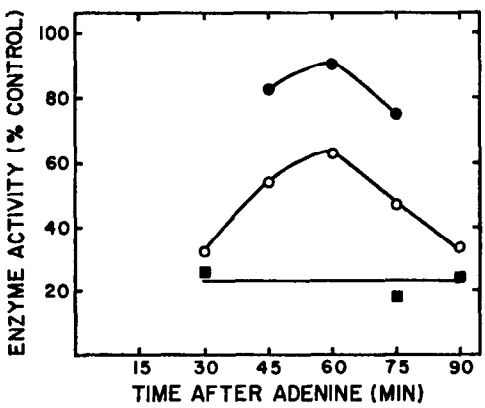

FIG. 1. Rate of recovery of thymidine kinase activity in azaserine-treated sarcoma 180 ascites cells after exposure to a source of preformed adenine. Mice bearing 6-day implants of sarcoma 180 ascites cells were injected i.p. with a single dose of 0·2 mg azaserine/kg. At the designated zero time, 11·5 hr later, a single dose of either 30 or 60 mg/kg of adenine was injected i.p. At intervals thereafter, cell-free extracts were prepared from sarcoma 180 cells and assayed for thymidine kinase activity. Each value represents the average of duplicate determinations from the pooled cell-free extracts of four mice. The enzyme from untreated cells converted 13·5 mμmoles thymidine/mg protein/hr. ■——■, thymidine kinase activity of azaserine-treated cells; ○——○, thymidine kinase activity of azaserine-treated cells exposed to 30 mg adenine/kg; ●——●, thymidine kinase activity of azaserine-treated cells exposed to 60 mg adenine/kg.

after the antibiotic was decreased to about 25 per cent of the level present in extracts from untreated cells. Adenine (30 mg/kg) partially restored thymidine kinase activity, which reached a maximum at 1 hr after exposure to the preformed purine and then declined. This decrease in enzyme activity probably reflected a depletion of the injected adenine and its derived nucleotides in the continued presence of blockade of the *de novo* purine nucleotide biosynthetic pathway by azaserine. With a larger amount of adenine (60 mg/kg), the activity of thymidine kinase in azaserine-treated cells was restored in 1 hr to levels which approximated those present in untreated cells.

The increase in thymidine kinase activity in azaserine-treated cells exposed to

adenine may be due to either: (1) stimulation of the synthesis of additional kinase molecules, or (2) activation of preexisting molecules of thymidine kinase by adenine or a derivative thereof. Puromycin and *p*-fluorophenylalanine were used in previous experiments,[16] prior to adenine-induced rescue of enzymatic activity in azaserine-treated cells, in an effort to distinguish between these alternatives. Although the findings were consistent with new protein synthesis, the results were ambiguous and did not allow a definite choice between the two possibilities. In an attempt to determine whether adenine or a nucleotide derivative thereof was capable of activating thymidine kinase, cell-free extracts from azaserine-treated cells were preincubated at 37° for 10 min with either adenine, adenosine 5′-phosphate or adenosine 5′-diphosphate employed at 10^{-4} M. Substrates were then added and thymidine kinase activity was measured. The low thymidine kinase activity of the cell-free extracts from azaserine-treated cells was not increased by any of the adenine derivatives. In other experiments, intact cells collected from azaserine-treated mice were disintegrated by sonication in 0·05 M Tris-Cl (pH 7·5) containing 0·05 mM of either thymidine, deoxycytidine 5′-diphosphate or adenosine 5′-triphosphate. The enzymatic activity obtained with the latter two nucleotides was no greater than that of extracts prepared in the presence of thymidine.

To test for the presence of an inhibitor of the activity of thymidine kinase in the cell-free extracts from cells exposed to azaserine, varying amounts of the extracts from azaserine-treated cells were mixed with extracts from untreated control cells. The resulting activity of thymidine kinase in each instance was that expected from the sum of the activities of the individual extracts, eliminating the possibility that (1) an inhibitor of thymidine kinase, formed from the treatment with the antibiotic, was present in the extracts of drug-treated cells, and (2) an activator of thymidine kinase destroyed by azaserine existed in enzymatic extracts of untreated cells. Such an experiment, however, does not eliminate the possible involvement of an inhibitor or activator which is tightly bound.

The relative abilities of hypoxanthine, thymidine and deoxycytidine to mimic the adenine-induced restoration of thymidine kinase activity in azaserine-treated cells were determined (Table 1). Administration of 60 mg hypoxanthine per kg to tumor-bearing mice 11·5 hr after azaserine and determination of enzyme activity 1 hr later indicated that hypoxanthine, a preformed purine capable of being converted to adenine nucleotides, was only weakly effective as a rescue agent. In contrast, thymidine or deoxycytidine, employed in an identical manner, was much more potent in this regard. Furthermore, a mixture of 60 mg per kg each of thymidine and deoxycytidine caused complete recovery of thymidine kinase activity.

The comparative effects of these agents on the size of the adenine nucleotide pool of sarcoma 180 ascites cells are shown in Table 2. A decrease of 61 per cent in the size of the intracellular pool of adenine nucleotides was caused by inhibition of purine nucleotide biosynthesis by azaserine. The utilization of preformed adenine for nucleotide formation allowed the cells to circumvent the block on the *de novo* purine nucleotide biosynthetic pathway. Thus, in the presence of a source of preformed adenine the adenine nucleotide pools returned to normal in azaserine-treated cells. In agreement with the weak activity of hypoxanthine in restoring thymidine kinase activity, only a slight rebuilding of the lowered adenine nucleotide pools was afforded by the availability to the cells of this purine, indicating that much of the administered

TABLE 1. EFFECTS OF PURINES AND PYRIMIDINE DEOXYRIBONUCLEOSIDES ON THE THYMIDINE KINASE ACTIVITY OF SARCOMA 180 ASCITES CELLS*

Pretreatment	Rescue compound (mg/kg)	Time after injection of rescue compound (hr)	Thymidine kinase activity (% control)
None	None	0·5 or 1	100
	Adenine, 60	1	104
	Hypoxanthine, 60	1	120
	Thymidine, 120	0·5	108
	Thymidine, 60 + deoxycytidine, 60	1	126
Azaserine	None	0·5 or 1	27
	Adenine, 60	1	74
	Hypoxanthine, 60	1	36
	Thymidine, 60	0·5	39
	120	0·5	49
	Deoxycytidine, 60	0·5	49
	120	0·5	67
	Thymidine, 60 + deoxycytidine, 60	0·5	102
		1	71

* Mice bearing 6-day implants of sarcoma 180 ascites cells were injected i.p. with a single dose of 0·2 mg azaserine/kg; 11·5 hr later, the indicated rescue compounds were injected i.p. and after 0·5 or 1 hr, the cells were collected and cell-free extracts were prepared and assayed for kinase activity. Each value represents the average of duplicate determinations from pooled cell-free extracts of four mice. The enzyme from untreated cells converted 13·5 mμmoles thymidine/mg protein/hr.

TABLE 2. EFFECT OF PURINES AND PYRIMIDINE DEOXYRIBONUCLEOSIDES ON THE SIZE OF THE ADENINE NUCLEOTIDE POOL OF AZASERINE-TREATED SARCOMA 180 ASCITES CELLS*

Pretreatment	Rescue compound (mg/kg)	Concn of adenine nucleotides	
		(μmoles/g cells)	(% control)
None	None	3·5	100
	Adenine, 60	3·7	105
	Hypoxanthine, 60	3·4	97
	Thymidine, 60 + deoxycytidine, 60	3·3	93
Azaserine	None	1·4	39
	Adenine, 60	3·1	88
	Hypoxanthine, 60	2·0	58
	Thymidine, 60 + deoxycytidine, 60	1·3	38

* Mice bearing 6-day implants of sarcoma 180 ascites cells were injected i.p. with a single dose of 0·2 mg azaserine/kg; 11·5 hr later, the indicated rescue compounds were injected intraperitoneally and 1 hr after either adenine or hypoxanthine, the adenine nucleotide pool was measured. In the case of the combined injection of thymidine and deoxycytidine, the pool was measured 0·5 hr later. Each value represents the average of the separate analyses of cells from four mice.

hypoxanthine was not anabolized, perhaps due to oxidation to uric acid. The mixture of thymidine and deoxycytidine, employed under conditions that returned the decreased enzyme levels to normal, did not affect the azaserine-depleted adenine nucleotide pools.

DISCUSSION

Maintenance of the size of intracellular pools of enzymic molecules is dependent upon the steady state imposed by the rate of synthesis of that particular protein, a process contingent, on one hand, upon gene multiplicity and the subsequent transcriptional and translational events and, on the other, upon the rate of enzymic breakdown. Several conceivable mechanisms for modulation of these processes exist; one of these influences resides in the capacity of substrates, cofactors and effector molecules to bind the enzyme and thereby presumably cause conformational changes in structure which serve to render the protein less susceptible to both proteolytic influences and natural decay in the cellular environment.[21] Consequently, metabolic alterations in the intracellular levels of substrates, cofactors and other molecules which interact with enzymic proteins, imposed either by the need for growth or function or by the stress of a toxic influence, can lower or raise the intracellular quantity of a particular enzyme.

The available evidence suggests that such a mechanism is involved in the decay of the activities of thymidine kinase and thymidylate kinase in sarcoma 180 cells treated with azaserine. Thus, it was hypothesized that kinase molecules are stabilized by the substrate ATP, an intracellular pool markedly depressed by treatment with azaserine.[16]

Thymidine kinase has been shown to be significantly protected against thermal inactivation *in vitro* by the substrates, thymidine and ATP, and the negative effector, deoxythymidine triphosphate;[22, 23] in addition, it also appears to be stabilized in intact cells by supplying these cells with thymidine.[24–27] The concept that the recovery of thymidine kinase activity in azaserine-treated cells subsequently exposed to adenine is due to the stabilization by ATP formed from adenine of newly synthesized molecules of thymidine kinase predicts that the other substrate of this enzyme, thymidine, will stabilize the enzyme in a conformation less susceptible to catabolic influences without altering the antibiotic-depleted adenine nucleotide pool. Such a result was indeed obtained, although thymidine was not as effective as adenine in facilitating recovery of thymidine kinase activity. Interestingly, a mixture of thymidine and deoxycytidine completely restored enzymic activity without affecting the size of the adenine nucleotide pool. Three alternatives appear to be available to explain this phenomenon. The first possibility requires an increase in the intracellular pool of deoxythymidine triphosphate derived from deoxycytidine; stabilization of thymidine kinase by deoxythymidine triphosphate may then occur in azaserine-treated cells supplied with deoxycytidine alone, and in those cells exposed to the antibiotic then subsequently treated with a mixture of deoxycytidine and thymidine. In the latter situation, enzyme conformation is envisioned to be fixed by reaction of thymidine with the active center of the enzyme and of deoxythymidine triphosphate with a feedback site. The second alternative requires the presence of a regulator site for a nucleotide derivative of deoxycytidine on the thymidine kinase molecule; consequently, both thymidine, by reaction with the active center of the enzyme, and deoxycytidine, after

conversion to a nucleotide, by binding to an effector site effectively stabilize the enzyme from degradative influences. Evidence for the presence of a regulatory site for deoxycytidine nucleotides was provided by Okazaki and Kornberg[28] and Iwatsuki and Okazaki[29, 30] who demonstrated, with a thymidine kinase enzyme from *Escherichia coli*, an activator site for dCDP and dCTP which, when charged with an activator, caused a dimerization of the enzyme to increase activity and stability against heat. Additional evidence was found by Bresnick *et al.*,[31] who showed that thymidine kinase from several transplanted rodent neoplasms was feedback inhibited by dCTP. The finding that deoxycytidine 5'-diphosphate did not alter the activity of thymidine kinase of sarcoma 180 when employed in the preparation of cell-free extracts suggests that such a mechanism is unlikely in the present situation. Last, since mouse tissues contain enzymes that can cleave thymidine to the base level (i.e. thymidine phosphorylase [thymidine: orthophosphate deoxyribosyltransferase; EC 2.4.2.4] and uridine phosphorylase [uridine: orthophosphate ribosyltransferase; EC 2.4.2.3],[32] it is conceivable that deoxycytidine enhanced the effectiveness of thymidine by protecting it from catabolic influences. This possibility, however, is made unlikely by the finding that deoxyuridine did not enhance the activity of thymidine in restoring thymidine kinase activity in azaserine-treated cells.

The intracellular levels of thymidine are relatively low in cells;[33] accordingly, the other substrate, ATP, would appear to be the most reasonable candidate for substrate-induced stabilization and modulation of the pool of thymidine kinase. Since the pool size of ATP is relatively large, it would seem that fine regulation of enzymic amounts by this nucleotide is impossible, unless a specific smaller pool is located in a compartment which houses this enzyme. That such a possibility may exist, however, was shown by the finding that sufficient levels of ATP were present in azaserine-treated cells to support the incorporation of orotic acid and leucine into RNA and protein respectively.

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