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Elevated Thymidine Phosphorylase Activity in the Plasma and Ascitic Fluids of Tumor-Bearing Animals[1] (40034)

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Neoplasia is often characterized by a qualitative and quantitative imbalance of key enzymes, particularly those regulating DNA and RNA synthesis (1, 2). Having defined more fully those metabolic alterations associated with tumors of a particular histopathology, these key enzymes will provide useful biomarkers for identifying and monitoring tumor progression, and will continue to be the major targets of different chemotherapy regimens (1).

We have recently identified thymidine (TdR) phosphorylase (EC 2.4.2.4; TdR: orthophosphate deoxyribosyltransferase) in the plasma of man and different laboratory animals (3), and have shown that patients with uncontrolled neoplastic diseases often exhibit levels of this pyrimidine nucleosidase that are significantly greater than those of healthy subjects (4). This enzyme catalyses the reversible reaction of TdR + orthophosphate $\rightleftharpoons$ thymine (Thy) + 2-deoxy-α-D-ribose-1-phosphate (5), and its presence in many normal and neoplastic cells of man and different animals as well as in various pathogenic and nonpathogenic prokaryotic cells has been well documented (5, 6). Some investigators have suggested that, under certain circumstances, this enzyme may play an important role in the salvage of Thy for nucleic acid synthesis in tissues having a high rate of proliferation; however, its physiological function, especially with respect to malignant tissues, has not been fully elucidated.

To further define the potential clinical and experimental utility of assaying TdR phosphorylase activity, a study of different tumor-bearing animals was undertaken. Reported herein are the results of experiments demonstrating that significant increases in this TdR-degrading enzyme were recorded in most of the tumor models tested.

Materials and methods. Adult rats and mice of different strains were used, and initial experiments were performed using animals that had been divided into 11 groups (Groups A–K, Table I). Each of these groups consisted of test animals having a tumor of a particular histological type and about an equal number of age- and sex-matched healthy control animals. The pathophysiology of these tumors and procedures used for their serial passage have been described elsewhere (7–13, see Table I).

Plasma was from fresh blood that had been collected asceptically into siliconized tubes containing EDTA (1.5 mg/ml) and sodium heparin (10 units/ml). Rat blood was collected by retroocular puncture and mouse blood was procured by decapitation. Ample blood from a single animal was obtained such that replicate enzyme determinations could be made using unpooled plasma samples. Ascitic fluid was aspirated from the peritoneal cavity with a syringe and needle. After performing a cell count, the fluid was centrifuged (250*g*, 8 min) and the resulting pellet containing tumor cells was washed twice with phosphate buffered saline (PBS, pH 7.2; 0.14 *M* NaCl, 4.0 m*M* KCl, 0.5 m*M* Na_2HPO_4 and 0.15 m*M* KH_2PO_4). Thereafter, the cells were resuspended to their original density in PBS and then disrupted by ultrasonication. With the exception of the mouse plasma, all samples to be assayed were passed through a micropore (0.22 μm) filter.

TdR phosphorylase activity was measured using our microassay (14); this is a modification of techniques that have been de-

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TABLE I. THYMIDINE PHOSPHORYLASE ACTIVITY IN THE PLASMA OF HEALTHY AND TUMOR-BEARING ANIMALS.

Group	Species (strain)	Tumor model	Animals assayed	No. of animals	% [^{3}H]TdR degraded (mean ± SE)	Significance (Student's *t* test)
A	Rat (Furth-Wistar)	Wilms' tumor (7)[a]	Healthy control	8	1.9 ± 0.3	$P < 0.001$
			Tumor-bearing	8	9.1 ± 0.6	
B	Rat (Charles River CD)	Zajdela ascites hepatoma (8)	Healthy control	8	2.6 ± 0.5	$P < 0.001$
			Tumor-bearing	8	17.5 ± 3.5	
C	Mouse (DBA/2)	L1210 lymphoid leukemia (9)	Healthy control	8	1.5 ± 0.3	$P < 0.001$
			Tumor-bearing	22	55.9 ± 7.2	
D	Mouse (RFM/Un)	Myeloid leukemia (10)	Healthy control	8	3.3 ± 0.4	$P < 0.001$
			Tumor-bearing	8	52.6 ± 4.0	
E	Mouse (A/Ha)	L#2 ascites lymphoma (11)	Healthy control	5	2.5 ± 0.5	$P < 0.01$
			Tumor-bearing	5	48.9 ± 13.2	
F	Mouse (Ha/ICR)	Ehrlich ascites tumor (9)	Healthy control	5	2.9 ± 0.9	$P < 0.05$
			Tumor-bearing	9	16.8 ± 1.6	
G	Mouse (Balb/c)	Neuroblastoma (9)	Healthy control	5	2.1 ± 0.2	$P < 0.005$
			Tumor-bearing	5	16.7 ± 3.3	
H	Mouse (BDF_1)	B16 melanotic melanoma (12)	Healthy control	6	2.3 ± 0.3	$P < 0.01$
			Tumor-bearing	6	8.9 ± 1.9	
I	Mouse (C3H/Bi)	Renal cell carcinoma (9)	Healthy control	9	2.3 ± 0.2	$P < 0.025$
			Tumor-bearing	9	6.7 ± 1.5	
J	Mouse (ICR × C57Bl/6Ha)	Gyn-74 uterine tumor (NR)[b]	Healthy control	10	3.7 ± 0.8	$P > 0.1$
			Tumor-bearing	11	5.4 ± 0.9	
K	Mouse (DBA/2)	Friend erythroblastic leukemia (13)	Healthy control	5	5.4 ± 1.3	$P > 0.4$
			Tumor-bearing	5	3.9 ± 1.2	

[a] Literature reference.
[b] Not reported.

scribed previously (15–17). In brief, 20 μl of plasma, ascitic fluid or sonic extract was added to a 200 μl U-shaped culture-well of a microtiter plate. To each culture-well was then added 20 μl of PBS containing 1.0 μCi [6-^{3}H] TdR (spec. act., 2.0 Ci/mmole; final conc., 12 m*M*). Immediately after mixing (time 0 hr, background samples) and after incubating at 37° for various times (usually 3.0 hr, test samples), 5 μl of the plasma–isotope mixture was spotted onto a Whatman No. 1 chromatography paper at a location that had been spotted previously with 5 μl of a saturated mixture of unlabeled TdR and Thy reference markers. Chromatography was performed as described previously (14–17) and radioactivity in grid sections containing the uv-absorbing TdR and Thy reference standards of each chromatography channel was then determined by liquid scintillation counting ($\cong 1 \times 10^5$ cpm; counting efficiency, 45 ± 0.1%). After subtracting background [^{3}H] Thy values (<3%), the amount of [^{3}H]TdR or [^{3}H]Thy present in each sample was defined as a percentage of the total radioactivity (amount recovered, ≥95%). Human plasma from patients showing high levels of TdR phosphorylase activity (4) were often included as standards for monitoring technical variability. These included aliquots of plasma which had (negative standard) or had not (positive standard) been heated (56°, 2 hr) to inactivate this enzyme before storing at −20°. Reproducibility among replicate assays was good (mean S.E., ≤5%). Enzyme activity was expressed as the percent [^{3}H]TdR degraded; this departure from conventional definitions of enzyme activity (i.e., product formation rather than substrate degradation) was justified in that it illustrated the potential magnitude of error that may arise in various *in vitro* or *in vivo* assays in which the cellular uptake of [^{3}H]- or [^{14}C]TdR is used as the sole basis for defining DNA synthesis (3, 4, 14–17).

TdR kinase activity was assayed using the method described by Lee and Cheng (18). The reaction mixture contained 0.19 *M* Tris-HCl (pH 7.5), 1.9 m*M* $MgCl_2$, 0.2 μCi [^{14}C]TdR (spec. act., 12 μCi/mmole; final conc., 0.19 m*M*), 1% bovine serum albumin, 3 mM phosphocreatine, 0.54 units creatinine kinase, 10 m*M* dithiothreitol, 10 m*M* NaF and test serum in a final volume of 0.1 ml. The assay mixture was usually incubated for 1.0–2.0 hr; however, in some assays, longer times were tested. The reaction was stopped by spotting 50 μl of the reaction mixture onto a Whatman DE81 disc, then immediately dropping it into alcohol (10 ml/disc) and washing three times with alcohol. Thereafter, the sample was prepared for liquid scintillation counting and the amount of [^{14}C]TMP formed was determined.

The results were subjected to statistical evaluation by means of the Student's *t* test for small samples, and differences between means giving a probability of less than 5% were considered to be significant.

Results. Summarized in Table I are the results of a survey defining TdR phosphorylase activity in the plasma of healthy and tumor-bearing rats ($N = 32$) and mice ($N = 131$). Significant ($P < 0.01$) differences in TdR phosphorylase activity were recorded for each of the two rat tumor models; in these experiments, enzyme activity in the plasma from animals with Wilms' tumor or Zajdela ascites hepatoma (Groups A and B) were 379 and 573% greater than the corresponding values for animals without tumors.

Significantly ($P < 0.05$) elevated TdR phosphorylase activity was also identified in 7 of the 9 (78%) murine tumor models examined. Of these, highest levels of enzyme activity were recorded for animals with lymphoid leukemia (Group C), myeloid leukemia (Group D) and ascites lymphoma (Group E). Increased activity was also recorded for mice with Ehrlich ascites tumor (Group F).

Of the four test groups with different types of solid tumors (Groups G–J), increased plasma TdR phosphorylase activity was noted in three (75%). Test mice failing to show an increase in TdR phosphorylase activity were those with a carcinogen-induced uterine tumor. This uterine tumor, which is currently being characterized, was transplanted subcutaneously and, when compared with other tumors in this study, was rather benign in that it showed little or no evidence of metastasis. Mice with Friend erythroblastic leukemia (Group K) also failed to show an increase in plasma TdR phosphorylase activity.

Presented in Table II are the results of a study comparing TdR phosphorylase activity in the plasma, ascitic fluids and tumor cell sonicates of mice with L1210 lymphocytic leukemia. This leukemia was transplanted intraperitoneally with 1.5×10^6 tumor cells and the animals were sacrificed 9 days later. Appreciable TdR phosphorylase activity was recorded in 0.5 hr reaction mixtures. Increased amounts of [^{3}H]TdR were degraded in reaction mixtures of 1.0–4.0 hr; mean values (±S.E., $N = 9$ mice) obtained in 2.0 hr reaction mixtures containing tumor cell sonicates, plasma and ascitic fluids were 45.0 ± 6.5, 31.8 ± 11.5 and 6.5 ± 1.6%, respectively.

In similar studies, corresponding mean values recorded in 3.0 hr assays of ascitic fluids from rats with Zajdela hepatoma (*N*

TABLE II. THYMIDINE PHOSPHORYLASE ACTIVITY IN THE PLASMA, ASCITIC FLUIDS AND TUMOR CELL SONICATES OF MICE WITH L1210 LYMPHOCYTIC LEUKEMIA.

Substance assayed	% [^{3}H]TdR degraded in reaction mixtures of:			
	0.5 hr	1.0 hr	2.0 hr	4.0 hr
Plasma	19.4 ± 9.5[a]	26.0 ± 11.4	31.8 ± 11.5	40.1 ± 11.4
Cell sonicates[b]	15.6 ± 2.2	27.5 ± 3.4	45.0 ± 6.5	61.6 ± 5.1
Ascitic fluids	3.2 ± 0.7	5.3 ± 1.7	6.5 ± 1.6	11.5 ± 2.6
Ascitic fluids-H[c]	0.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.4	1.3 ± 0.6

[a] Mean ± SE, $N = 9$ mice.
[b] Cell density (mean ± SE) before lysis, $12.7 \pm 0.6 \times 10^7$/ml PBS.
[c] Heated (H) at 56° for 2.0 hr.

= 10), mice with L#2 lymphoma and mice with Ehrlich tumor (N = 13) were 19.2 ± 1.7, 30.3 ± 10.1 and 29.2 ± 5.9, respectively.

Presented in Fig. 1 are the results of a study comparing changes in plasma TdR phosphorylase activity with tumor progression in mice with myeloid leukemia. Parameters of tumor growth which were monitored included changes in the spleen weight, nucleated spleen cells, liver weight and blood leukocytes. Enzyme activity and these different parameters were analyzed for mice transplanted intravenously with 1.0×10^6 splenic leukocytes from a leukemic mouse. Groups of animals (N = 3/group) were sacrificed on days 0 (i.e., immediately after tumor transfer), 1, 4 and 8. There were no major changes within the first day. On day 4, however, we detected a significant increase in TdR phosphorylase activity that was accompanied by splenomegaly. These changes, as well as the concomitant development of hepatomegaly, became more pronounced on day 6. On day 8, a time at which the mice were in the terminal phase of their disease, highest values were recorded for TdR phoshorylase activity and for each of the four morphological parameters.

Shown in Fig. 2 are the results of a representative study of TdR kinase and TdR

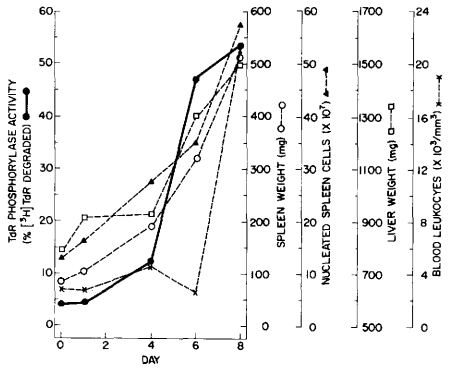

FIG. 1. Comparison of different morphological parameters of tumor progression with thymidine phosphorylase activity in the plasma of mice having myeloid leukemia. Assays were performed immediately after tumor transplantation (day 0) and at various times thereafter. Each point denotes the mean value of three animals. Reproducibility of the different assays was good, usually less than 15% SE.

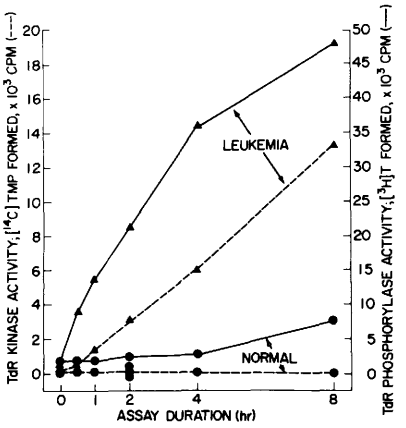

FIG. 2. Analysis of TdR kinase and TdR phosphorylase activities in the sera of a test mouse in the terminal stage of his myeloid leukemia and of a corresponding healthy control animal. Each point denotes the mean value of triplicate assays, SE ≤ 7%.

phosphorylase activities in the sera of a moribund mouse with myeloid leukemia and of a healthy animal of the same strain, age and sex. Significant ($P < 0.001$) increases in both of these enzymes were recorded for the leukemic mouse, and activities of these enzymes were nearly linear in 4.0 hr reactions. Values for the corresponding enzymes in the sera of the normal mouse were consistently low.

Discussion. Selected for initial experimentation were animals having tumors that differed markedly with respect to their strain specificity, etiology, histology, growth, invasiveness and morbidity. Results of these studies demonstrated that TdR phosphorylase activity in the plasma of tumor-bearing animals was increased significantly in 9 of 11 (82%) models tested.

The findings of experiments reported herein are consistent with the observations of our previous report showing that the activity of this TdR-degrading enzyme is frequently elevated in the plasma of patients with uncontrolled neoplastic diseases who had or had not received antitumor therapy (4). Accordingly, increased plasma TdR phosphorylase activity in the tumor-bearing patient cannot be ascribed solely to impaired cell function resulting from chemotherapy, radiotherapy, surgery or other antitumor modalities. Moreover, it is unlikely that elevated activity of this extracellular

enzyme can be attributed to undetected viral, bacterial or fungal infections. Thus, changes in TdR phosphorylase activity noted in these tumor-bearing hosts are thought to be associated with tumor growth. Equally important, however, is that studies reported herein have identified several tumor models suitable for future experiments characterizing the origin, function and kinetics of this enzyme.

The reasons for this increased activity of TdR phosphorylase are not known, and we cannot exclude as possible explanations the release of TdR by rapidly replicating tumor cells, by tumor tissue necrosis and disoluton, or by normal tissues whose function has been altered either directly by metastatic infiltration or indirectly by humoral factors.

In previous studies attempting to define the mechanisms whereby TdR phosphorylase is released, we have shown that high levels of this enzyme are elaborated by lymphoblastoid cells of established human lines when these cells were maintained for a short time under certain culture conditions having no apparent adverse affect on their viability or morphology (14). Other *in vitro* experiments have shown that TdR phosphorylase is also released by peripheral blood leukocytes of healthy donors as well as of patients with either myeloid or lymphoid leukemia (14–19). These and other studies currently in progress suggest that the extracellular presence of TdR phosphorylase is not merely a consequence of cell death, and that it may be selectively released as are the lysosomal enzymes of polymorphonuclear leukocytes (21). In this respect it is noteworthy that the polymorphonuclear leukocyte is thought to contain significantly higher levels of TdR phosphorylase than mononuclear leukocytes (15–17).

The biochemical strategy of cancer cells, particularly with respect to the imbalance of enzymes of pyrimidine synthesis and degradation and in the *de novo* and salvage pathways of DNA, have been studied extensively. Of particular interest have been the findings of Weber and co-workers who have formulated the "molecular-correlation concept" (1, 2). Essential to this idea is the existence of certain enzymes whose concentrations and activities increase, and those of opposing enzymes that decreased, with malignant transformation or malignant progression (1, 2). Examination of homogenates or sonic extracts of different types of tumors as well as of tumors having a similar histology but varied growth rates has provided evidence suggesting that intracellular TdR phosphorylase activity is decreased in most tumors during periods of accelerated growth. The reason for the apparent discrepancy between the activity of intracellular and extracellular TdR phosphorylase activity cannot readily be resolved with data that are currently available.

Assuming that the "molecular-correlation concept" is correct, an interpretation of our findings of increased TdR phosphorylase activity in the tumor-bearing host is further complicated by information from recent publications reporting increased tumor-associated TdR kinase activity in the sera of rats with transplantable hepatomas (19) and lymphomas (20), but not of hosts carrying hepatic (observations conflict with those of reference 19), renal, mammary or submaxillary gland tumors (20). In addition to the studies of mice with myeloid leukemia (see Fig. 2), we have also observed increased TdR kinase activity in the sera of other animals with transplanted tumors including rats with hepatomas (unpublished). This apparent biochemical imbalance of TdR phosphorylase and TdR kinase, enzymes of the pyrimidine pathway that are functionally opposed to one another (1, 2), requires further characterization in serial studies correlating the activities and concentrations of these enzymes in both tumor cells and plasma with tumor progression and the pathophysiology of the host.

Summary. Activity of thymidine (TdR) phosphorylase (EC 2.4.2.4; TdR: orthophosphate deoxyribosyltransferase) in the plasma of healthy and tumor-bearing mice and rats was evaluated in comparative studies. Increased enzyme activity was recorded in 9 of 11 tumor models tested, and highest values were obtained for mice with lymphoid or myeloid leukemia. Heightened activity of this TdR-degrading enzyme was observed in the plasma of mice bearing three of four solid tumors. Elevated TdR phos-

phorylase activity was also identified in the peritoneal exudate fluids in each of four groups of animals carrying ascites-producing tumors.

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