

# Thymidine Kinase Isoenzymes in Human Malignant Lymphoma<sup>1</sup>

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## ABSTRACT

The activities of thymidine kinase (TK) isoenzyme 1 and 2 were examined in extracts of human benign or malignant lymphoid tissue and correlated with degrees of morphological differentiation. TK2 activity occurred in peripheral blood lymphocytes of normal individuals, patients with chronic lymphocytic leukemia, or solid lymphoid tissue, exhibiting either non-neoplastic histological findings or those of diffuse well-differentiated lymphocytic lymphoma. TK1 activity occurred in solid, non-Hodgkin's lymphoma tissue, exhibiting lesser degrees of cellular differentiation, or in peripheral blood lymphocytes of patients with clinical aggressive chronic lymphocytic leukemia or lymphosarcoma leukemia. In non-Hodgkin's lymphoma tissue, the range of TK1 activities correlated broadly with the Rappaport classification, with higher values occurring in tissue exhibiting changes of diffuse poorly differentiated lymphocytic lymphoma or diffuse histiocytic lymphoma.

## INTRODUCTION

The clinical prognostic value of the histological classification of NHL<sup>4</sup> proposed by Rappaport is generally accepted (8, 18, 30), but because its formulation predated the technology for immunological identification of neoplastic cells it has been criticized for its biological inaccuracy (3, 10, 16). More recently, Lukes and Collins (24, 25) have proposed that human malignant lymphoproliferative disorders can be recognized morphologically as having B- or T-cell origin, a premise based in part on the similarities between morphological changes for mitogen-transformed lymphocytes, with those for the reactive lymphoid follicle, and cytology of malignant lymphoma cells.

The addition of a mitogen such as PHA to human lymphocytes *in vitro* results in a series of cellular events culminating with cellular division. Among the myriad biochemical events accompanying this blastogenesis is an increase in the activity of the pyrimidine salvage pathway enzyme TK (ATP:thymidine 5'-phosphotransferase, EC 2.7.1.21) which catalyzes the phosphorylation of thymidine to TMP (2, 27). In human tissue, this activity occurs as 2 isoenzymes termed TK1 and TK2 (31). The activity of TK1 is associated with the cytoplasmic cell fraction and migrates slowly during polyacrylamide electrophoresis; it differs from TK2 which is associated with the mito-

chondrial cell fraction and migrates rapidly during polyacrylamide electrophoresis (20, 32). The 2 forms also differ in sedimentation coefficient, pH optimum, phosphate donor specificity, and inhibition by dCTP (5, 20, 21, 32). With cellular proliferation, it is the activity of TK1 that parallels changes in DNA synthesis, while that of TK2 remains relatively constant (1, 19).

For these reasons, we decided to study the profile of TK isoenzyme activities in human lymphoid tissue of various lymphoproliferative disorders, particularly NHL, to determine whether a relationship existed between isoenzyme activity status and histological findings according to criteria proposed by Rappaport with their established clinical implications (8, 18).

## MATERIALS AND METHODS

**Materials.** Thymidine, purine and pyrimidine nucleotides, acrylamide, bisacrylamide, and dithiothreitol were obtained from Sigma Chemical Company, St. Louis, Mo. [6-<sup>3</sup>H]Thymidine (5 and 18.9 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. All other chemicals used were of analytical grade and were from commercial sources.

**Patients Studied.** Peripheral blood lymphocytes were obtained from 14 hematologically normal individuals and 17 patients with nonneoplastic medical disorders; these comprised the control group. Patients studied with lymphoproliferative disorders included 12 with CLL and 4 with LCL. The group with CLL all exhibited an absolute lymphocytosis with the majority of peripheral blood lymphocytes exhibiting faint immunofluorescence<sup>5</sup> for monoclonal IgM or IgM-IgD; patients with LCL had NHL together with the presence of pleomorphic lymphoblastoid cells in the peripheral blood, exhibiting bright immunofluorescence for monoclonal IgG (15). Solid lymphoid tissue was obtained at surgical biopsy in 44 individuals, and histological findings were independently reviewed by G. Medley. In 12 individuals, the findings were either normal or compatible with nonneoplastic inflammatory or hyperplastic changes; these patients formed the control group. The remaining 32 patients showed findings of NHL categorized according to the Rappaport classification with the modification of Berard and Dorfman (4). Individual categories were: DWDLL, 1; DILL, 6; NPDLL, 7; DPDLL, 9; and DHL, 9.

**Cell Culture.** Normal human peripheral blood lymphocytes were obtained by density centrifugation of heparinized blood on Ficoll-Hypaque, and the cells obtained were cultured in triplicate with PHA as described previously (17, 33). At 20, 44, and 68 hr in culture, 1  $\mu$ Ci of [6-<sup>3</sup>H]thymidine (18.9 Ci/mmol) was added to one set of cultures; after incubation for 4 hr, radioactivity in trichloroacetic acid-precipitated material was assayed in a liquid scintillation counter (17). Cells in the resid-

<sup>5</sup> Lymphocyte immunofluorescence studies were kindly performed by members of the Clinical Immunology Unit, Peter MacCallum Clinic, Melbourne, Australia.

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<sup>4</sup> The abbreviations used are: NHL, non-Hodgkin's lymphoma; PHA, phytohemagglutinin; TK, thymidine kinase; CLL, chronic lymphocytic leukemia; LCL, lymphosarcoma cell leukemia; DWDLL, diffuse well-differentiated lymphocytic lymphoma; DILL, diffuse intermediate differentiated lymphocytic lymphoma; NPDLL, nodular poorly differentiated lymphocytic lymphoma; DPDLL, diffuse poorly differentiated lymphocytic lymphoma; DHL, diffuse histiocytic lymphoma.

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ual cultures were harvested at 24, 48, and 72 hr and assayed for TK activity as described below.

**TK Assay.** TK activity was determined in peripheral blood lymphocytes of normal individuals and patients with CLL or LCL. Enzyme extracts were prepared from cells harvested either from peripheral blood by Ficoll-Hypaque centrifugation or following culture with PHA as described previously (11, 17). Lymphoid tissue obtained at biopsy was divided into aliquots for histological examination and TK activity determination. Tissue samples (0.5 to 1.5 mg, wet weight) were rinsed twice with cold 0.9% NaCl solution and then homogenized twice in 2 volumes (w/v) of 50 mM Tris HCl (pH 7.4) containing 1 mM EDTA using a VirTis 45 homogenizer (VirTis Co., Gardiner, N. Y.) at a setting of 15 in four 20-sec bursts. The homogenate was centrifuged at 4° for 15 min at 10,000 × g, and the supernatant was removed for assay.

TK was assayed as described previously using [6-<sup>3</sup>H]thymidine as the radiolabeled substrate, with either ATP (5 mM) or CTP (5 mM) as the phosphate donor, and the ratio of CTP-dependent activity to ATP-dependent activity was determined (11, 12). Protein was determined according to the method of Lowry *et al.* (23) using bovine serum albumin as a standard, and enzyme activity was expressed as nmol per mg protein per hr.

**Thymidine Affinity Gel Chromatography.** The thymidine-Sepharose affinity gel was prepared essentially according to the method of Kowal and Markus (22). Two-ml aliquots of extracts of peripheral blood lymphocytes or solid lymphoid tissue prepared as described above were applied to the thymidine affinity column (0.5 × 4.5 cm), and the enzyme activity was eluted with increasing thymidine concentrations as described previously (12). For characterization of TK activity obtained with thymidine affinity chromatography of tissue extracts, the degree of enzyme inhibition by dCTP (2 mM) and TTP (2 mM) and activity at acid pH by substituting 50 mM sodium acetate buffer (pH 5.0) for 50 mM Tris-HCl (pH 7.4) were determined. In these experiments, the concentration of ATP and CTP was 2 mM.

**Polyacrylamide Gel Electrophoresis.** Electrophoresis of TK activity in tissue extracts was performed in 5% polyacrylamide gels according to the method of Kit *et al.* (21) with minor modifications (11).

## RESULTS

**Characterization of TK Activity in Normal and Neoplastic Lymphoid Tissue.** A one-step purification procedure utilizing thymidine affinity chromatography was used to characterize the TK activity occurring in normal or malignant lymphoid tissue. Chart 1 shows representative activity elution profiles of normal and malignant tissue extracts and 2 peaks of TK activities, arbitrarily designated as Peak 1 eluting with 0.2 M Tris-HCl buffer (pH 7.4) containing 100 μM thymidine and Peak 2 eluting with 0.4 M Tris-HCl buffer (pH 7.4) containing 300 μM thymidine, were consistently identified. Peak 1 and 2 activities were examined for comparative properties known to distinguish TK1 and TK2 (5, 20, 21, 32). With histological normal lymphoid tissue or CLL peripheral blood lymphocytes, both peaks showed properties consistent with those of TK2. The activity with CTP (2 mM) was 86 to 93% of that observed with equimolar ATP; TTP (1 mM) and dCTP (1 mM) produced mean percentage

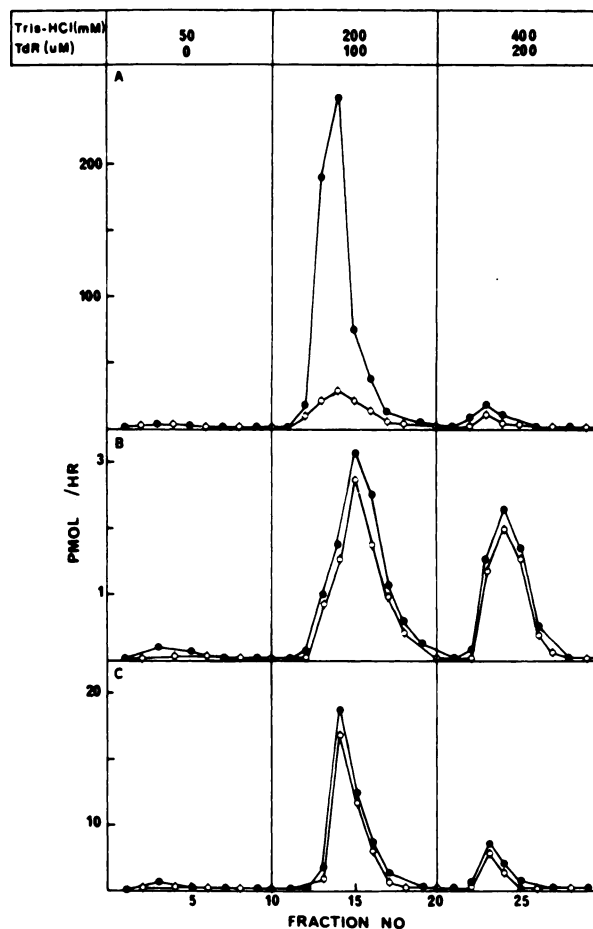


Chart 1. Elution activity profiles of TK of normal and neoplastic lymphoid tissue chromatographed on a thymidine-Sepharose affinity column as outlined in the text. ●, TK activity with ATP (5 mM) as the phosphate donor; ○, TK activity with CTP as the phosphate donor. (5 mM). A, diffuse histiocytic lymphoma lymph node extract; B, CLL peripheral blood lymphocyte extract; C, normal lymph node extract. TdR, thymidine.

inhibitions of ATP-mediated activity of 88 and 64%, respectively, and enzyme activity at pH 5.0 was 85% of that observed at pH 7.4. The corresponding peaks obtained with affinity chromatography of DHL extracts showed differential properties consistent with TK1 for Peak 1 and TK2 for Peak 2. With Peak 1 preparations, TK activity with CTP as the phosphate donor was 15% of that observed with ATP; dCTP produced 20% inhibition of ATP-mediated activity, and enzyme activity at pH 5.0 was 50% that observed at pH 7.4. TK from extracts prepared from histologically normal lymphoid tissue or that of DWLL and NPDLL were compared by polyacrylamide gel electrophoresis (Chart 2). Normal and DWLL extracts showed a single form of fast-moving activity ( $R_f$  0.44) which utilized ATP and CTP equally well as the phosphate donor. Enzyme recovery was 50 to 60%. Extracts of NPDLL tissue showed, besides activity remaining at or near the origin, 2 distinct forms of the enzyme at  $R_f$  0.25 and 0.40 with the activity occurring either at the origin or at an  $R_f$  of 0.25 utilizing as the phosphate donor ATP more efficiently than CTP, in contrast to that observed with the fast-moving form of TK ( $R_f$  0.40). Enzyme recovery was 25 to 30%.

**TK Activities in Unstimulated and PHA-stimulated Peripheral Blood Lymphocytes and Benign Solid Lymphoid Tissue.**

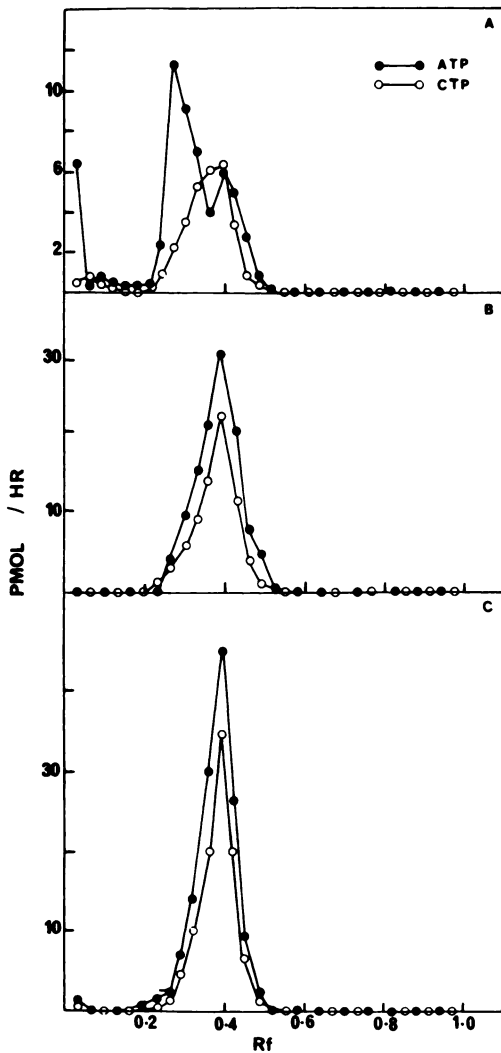


Chart 2. Electrophoretic patterns of TK activity from normal and neoplastic lymphoid tissue. Tissue extracts were prepared and electrophoresed as outlined in the text. Gel fractions were assayed for TK activity with either ATP (●) or CTP (○) as the phosphate donor. A, NPDLL lymph node extract; B, DWDLL lymph node extract; C, normal lymph node extract.

The findings presented above suggested that the ratio of enzyme activity obtained with either CTP or ATP as the respective phosphate donor was a useful means to differentiate between predominance of either TK1 or TK2 isoenzyme in tissue extracts. To facilitate the investigation of the nature of TK in malignant lymphoproliferative disorders, the range of activities and ratio of CTP-dependent activity to ATP-dependent activity was established for normal lymphoid tissue. Peripheral blood lymphocytes from normal individuals and patients with non-malignant medical conditions showed TK activities of  $0.18 \pm 0.06$  (S.D.) nmol/hr/mg protein with a ratio of CTP activity to ATP activity of  $0.71 \pm 0.10$ . With PHA-stimulated lymphocytes, the activity of TK increased at 48 hr to  $0.85$  nmol/hr/mg protein and at 72 hr it increased to  $2.8$  nmol/hr/mg protein with corresponding CTP/ATP ratios of  $0.12$  and  $0.03$ , respectively. With extracts prepared from histologically benign lymphoid tissue, the TK was  $0.14 \pm 0.05$  nmol/hr/mg protein with an ATP/CTP ratio of  $0.71 \pm 0.10$ .

**TK Activity in NHL Tissue and CLL or LCL Lymphocytes.** The profiles of TK activities in NHL tissue classified according

to the histological categories of Rappaport are shown in Chart 3. The single instance of DWDLL studied showed an activity of  $0.49$  nmol/hr/mg protein with a CTP/ATP ratio of  $0.60$ . The histological findings were that of a population of small mature lymphocytes. TK activities of lymphoid tissue of 6 patients with DILL ranged from  $0.18$  to  $1.0$  (mean,  $0.50$ ) nmol/hr/mg protein with a mean CTP/ATP ratio of  $0.22$  (range,  $0.10$  to  $0.25$ ). Histological examination of these tissues showed a more pleomorphic immature population of lymphocytes with increased mitotic figures. For the histological subtypes of NPDLL and DPDLL, TK activities ranged from within the range exhibited by benign lymphoid tissue to, respectively, 7 and 30 times these values. For NPDLL, the range of TK activity was  $0.15$  to  $1.1$  (mean,  $0.44$ ) nmol/hr/mg protein with a mean CTP/ATP ratio of  $0.18$  (range,  $0.14$  to  $0.24$ ); that for DPDLL ranged from  $0.19$  to  $5.7$  (mean,  $1.3$ ) nmol/hr/mg protein with a mean CTP/ATP ratio of  $0.22$  (range,  $0.17$  to  $0.27$ ). The TK activities exhibited by DHL tissue were consistently in excess of the control range, and a 17-fold variation was found within this subcategory (Chart 3) ranging from  $0.5$  to  $8.5$  (mean,  $3.5$ ) nmol/hr/mg protein with a mean CTP/ATP ratio of  $0.14$  (range,  $0.11$  to  $0.2$ ). Histological findings in this group were typical for DHL, namely, the presence of an intense pleomorphic dedifferentiated population of lymphoblasts and frequent mitotic figures.

Peripheral blood lymphocytes from 12 patients with CLL exhibited TK activities ranging from  $0.1$  to  $0.25$  (mean,  $0.16$ ) nmol/hr/mg protein with a mean CTP/ATP ratio of  $0.80$  (range,  $0.7$  to  $0.9$ ), and the morphological characteristics of these cells were predominantly of the small mature lymphocyte characteristic of CLL. In 4 patients, TK activity in peripheral blood lymphocytes disclosed a reduced ATP/CTP ratio of  $0.3$  (range,  $0.21$  to  $0.35$ ); in 2 individuals, this low ratio was found

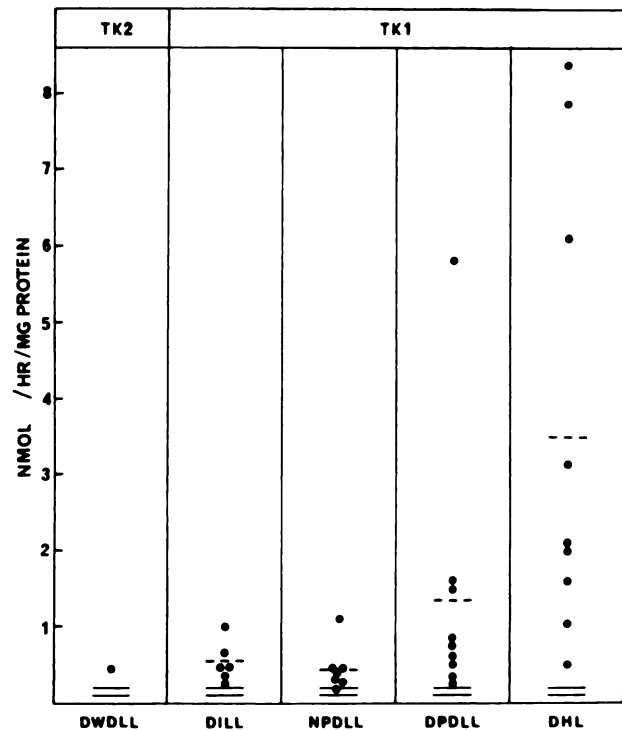


Chart 3. TK activity and isoenzyme type in NHL lymphoid tissue categorized according to the modified Rappaport classification. —, range in histologically benign lymph node extracts; ---, mean activity in each subgroup.

on initial clinical presentation and the other 2 patients' activities reverted from a high to low CTP/ATP ratio during course of their disease. TK activities of these patients ranged from 0.20 to 1.50 (mean, 0.62) nmol/hr/mg protein, and in all 4 patients the cytological appearances of peripheral blood mononuclears showed a pleomorphic subpopulation resembling prolymphocytes and lymphoblasts. Peripheral blood lymphocyte extracts of the 4 patients with LCL showed TK activities with a low CTP/ATP ratio of 0.2 (range, 0.15 to 0.25), the activities ranging from 0.20 to 3.50 (mean, 1.9) nmol/hr/mg protein. The mean TK activities and ATP/CTP ratios of normal and neoplastic peripheral blood lymphocytes or solid lymphoid tissue activities are summarized in Table 1.

## DISCUSSION

In this study, the comparison of the ratio of CTP-dependent TK activities to ATP-dependent TK activities in normal and neoplastic tissue with properties exhibited with either thymidine affinity chromatography or polyacrylamide gel electrophoresis suggested that this ratio was a useful index for determining the predominant presence of TK1 or TK2 isoenzymes. This premise was supported by the high CTP or ATP ratio of enzyme activities, indicative of the TK2 isoenzyme in nondividing normal peripheral blood lymphocytes and a progressive reduction in this ratio *pari passu* with induction of TK1 isoenzyme following PHA stimulation. Inasmuch as TK2 is also a deoxycytidine kinase (9), the relative activities with either substrate are another potential index to distinguish TK isoenzymes in tissue extracts. Because of the known catalytic lability of TK1, it should be emphasized that this activity is underestimated in tissue extracts and that the development of more sophisticated techniques such as enzyme radioimmunoassay is required to confirm the apparent lack of this isoenzyme by catalytic assays.

The profile of TK activities in NHL tissue correlated broadly with histological findings classified according to the Rappaport scheme as modified by Berard and Dorfman (4). In DWLL and CLL, the leukemic phase of DWLL (28), the finding in peripheral blood lymphocytes of TK2 activities was accompanied by mature cytological features and the known low proliferative status of those cells (26). In contrast, TK1 activity in CLL was accompanied, although not in all patients, by cells in the peripheral blood resembling prolymphocytes and frank lymphoblasts. Of particular interest was the conversion of TK isoenzyme status with change from clinical indolent to aggressive disease, a well-documented clinical phenomenon usually accompanied by the appearance of a peripheral blood population of immature cells resembling prolymphocytes and frank immunoblasts with an enhanced *in vitro* [<sup>3</sup>H]thymidine incorporation (7, 13, 26). In this setting, the appearance of TK1 activity as disclosed in this study could well be a biological probe for clinically aggressive disease, a hypothesis supported by the finding of TK1 activity in peripheral blood cells in patients with LCL, the disseminated accelerated phase of NHL (15).

Histological and immunological variability is a well-documented phenomenon in NHL (4, 6, 29). The increase in mean TK1 activities (Table 1) in tissue of patients with NPDLL through DPDL to DHL correlates with the known morphological cellular dedifferentiation accompanying this progression (4). That TK isoenzyme status may be a useful probe for cellular differentiation and proliferation is suggested by the difference of isoen-

Table 1  
Mean TK and ratio of CTP- and ATP-mediated activity in normal and neoplastic lymphoid tissue

Tissue	TK activity (nmol/hr/mg protein)	CTP/ATP ratio
Peripheral blood lymphocytes		
Control (n = 31)	0.18	0.71
Indolent CLL (n = 12)	0.16	0.80
Aggressive CLL (n = 4)	0.62	0.30
Lymphosarcoma cell leukemia (n = 4)	1.9	0.20
Solid lymphoid tissue histological status		
Nonneoplastic (n = 12)	0.14	0.71
Diffuse well-differentiated lymphocytic (n = 1)	0.49	0.60
Diffuse intermediate differentiated lymphocytic (n = 6)	0.50	0.22
Nodular poorly differentiated lymphocytic (n = 7)	0.44	0.18
Diffuse poorly differentiated lymphocytic (n = 8)	1.3	0.22
Diffuse histiocytic (n = 9)	3.5	0.14

zyme in DILL and DWLL, the subcategory of NHL with which it is most often categorized (14). The TK1 status of DILL was compatible with the population of larger, cytologically less mature lymphocytes and increased mitotic figures found in this category (14). The stepwise increase in mean TK1 activities for NPDLL through DPDL to DHL tissues indicates that this activity may well also be a useful parameter for predicting the clinical behavior of these tumors. Indeed, the observed variation of these activities within each of these subgroups suggests that this probe for clinical aggressiveness may in fact apply not only for these subgroups as a whole but also for the individual patient. Further studies addressing this question are currently under evaluation in this laboratory.

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