Terminal Deoxynucleotidyl Transferase as a Biological Marker for Human Leukemia

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Abstract

High levels of terminal deoxynucleotidyl transferase have been observed in leukocytes of 7 out of 20 patients with chronic myelogenous leukemia in acute blast phase of the disease. These levels are comparable to the levels observed in human and calf thymus gland and cell lines with some T cell characteristics (Molt 4 and 8402). Negligible levels of this activity were observed in chronic myelogenous leukemia not in an acute blast phase of the disease, chronic lymphocytic leukemia, human B cells, mature T cells, and the mixed population of lymphocytes present in normal human blood. The detection of this enzyme in some patients with chronic myelogenous leukemia in acute blast phase of the disease suggests that the blast proliferation may involve primitive stem cells which have more lymphoid than myelogenous characteristics. This enzyme assay may be of use as a biological marker for following patients during treatment and in remission.

Introduction

Terminal deoxynucleotidyl transferase, an enzyme that catalyzes the polymerization of deoxyribonucleotides onto the 3'-OH ends of oligo or polydeoxynucleotide initiators in the absence of a template was initially considered to be specific for the thymus gland (1). Recently, this enzyme has been detected in various forms of human leukemia (2–7) including acute lymphocytic leukemia (ALL) (2, 6, 7), acute myelomonocytic leukemia (AMML) (3), and chronic myelogenous leukemia (CML) in acute blast phase of the disease (5, 6, 7). High levels of this enzyme have also been observed in cell lines with T-cell characteristics (Molt 4 and 8402) derived from cells of patient with acute lymphoblastic leukemia (8, 9). The presence of high levels of this enzyme in cells from patients with chronic myelogenous leukemia in acute blast phase of the disease and its absence in the chronic phase of the disease (6, 7) suggest that this enzyme can be useful as a biological marker for following patients during treatment and in remission.

In this report we present a comparison of the levels of this enzyme in leukocytes of patients with chronic myelogenous leukemia in acute blast phase with the levels present in leukocytes from patients with CML not in acute blast phase, ALL, chronic lymphocytic leukemia (CLL), normal blood lymphocytes, human and

calf thymus gland and cell lines with B and T cell characteristics. In addition, we show that in leukocytes of a CML patient in remission, levels of this enzyme returned to the level present in normal leukocytes.

Materials and Methods

Materials: Tritium labeled deoxyribonucleoside triphosphates were obtained from Schwarz-Mann. Unlabeled deoxyribonucleoside triphosphates were obtained from P. L. Biochemicals. Oligo- and poly-ribo and deoxyribo-nucleotides were obtained from Miles Laboratories and P. L. Biochemicals.

Terminal transferase assays: Terminal transferase activity was assayed at 37° for 1 hr as described earlier (5, 6, 8) in a standard reaction mixture (0.05ml) which contained 50 mM Tris HCl (pH7.5), 50 mM KCl, 0.1 mM $MnCl_2$, 5 mM dithiothreitol (DTT), 100 μM of the labeled deoxyribonucleoside triphosphate, 2.5 μg of poly(dA) as a primer, and 5 μl of the enzyme fraction. The specific activity of the labeled deoxyribonucleoside triphosphates used was [^{3}H] dGTP (1500 cpm/pmole) and [^{3}H]dTTP (2200 cpm/pmole). The reaction was arrested by the addition of 50 μg of yeast tRNA and 2 ml of 10 % trichloracetic acid, collected on Millipore filters and counted in a scintillation counter (10).

Source of cells: White blood cells from leukemic patients were collected by the use of an IBM white cell separator (11). Lymphoblast cell lines (Molt 4 and 8402) with T cell characteristics, established in tissue culture were originally derived from peripheral blood of patients with ALL (12). All these cell lines were obtained from Hem Research Associates, Bethesda, Maryland. Another B cell line (NC37) was derived from human normal blood lymphocytes (13) and was obtained from J. L. Smith Memorial for Cancer Research, Pfizer, Inc., Maywood, New Jersey. Fresh human blood lymphocytes were obtained by phlebotomy from normal subjects purified from other blood components, and stimulated with phytophemagglutinin for 72 hrs. as described earlier (14). Fresh human thymus, obtained from children undergoing cardiac surgery, and calf thymus were obtained from Hem Research Associates, Bethesda, Maryland.

Cytogenetic studies on the cells of patients with chronic myelogenous leukemia in chronic and acute blast phase showed the presence of philadelphia chromosome (Ph_1).

Cell extractions: All cell extractions were carried our at 0–4 °C. Cells were washed with phosphate buffered saline (pH 7.4) twice, and suspended in 5 volumes of buffer A (10mM Tris.HCl (pH 7.4), 10 mM KCl and 1.5 mM Mg^{2+}) and allowed to swell for 10 min. The cells were manually disrupted in a tight fitting stainless steel homogenizer and mixed with equal volume of buffer B (50 mM Tris.HCl (pH 7.5), 5 mM DTT, 1 M KCl, 1 % triton X100, 20 % glycerol) and stirred in ice for 2 hrs. The soluble extract was removed, dialyzed against buffer C (50 mM Tris. HCl (pH 7.5), 5 mM DTT, 20 % glycerol) containing 0.5M KCl and subsequently against buffer C. Human and calf thymus gland was processed according to an earlier procedure (6).

Enzyme purification: Terminal transferase was purified by successive chromatography on DEAE cellulose, phosphocellulose and hydroxyapatite as described earlier (5, 6, 8).

Results

Terminal transferase levels in various cells. High levels of terminal transferase were observed in cells of some patients with chronic myelogenous leukemia in acute blast phase of the disease. These levels are comparable to the levels of terminal transferase observed in lymphoid cell lines with T cell characteristics such as Molt 4 and 8402 (6, 8, 9) and human and calf thymus gland (Table 1). Insignificant levels of terminal transferase were observed in B cell lines (NC37, SB and 8392), PHA stimulated normal human blood lymphocytes, chronic myelogenous leukemia not in an acute blast phase, chronic lymphocytic leukemia and acute myelogenous leukemia, except one AML patient whose cells contained low but definite terminal transferase activity (Table 1). Table 2 summarizes our results on the detection of terminal transferase in the cells of a number of leukemic patients. As shown in this table, we have observed high levels of terminal transferase in 7 out of 20 patients with chronic myelogenous leukemia in acute

Table I: Terminal transferase activity in normal and malignant cells*

Source of Cells	Diagnosis	Incorporation of [^{3}H]dGMP (nmoles per hr per 10^8 cells)
1. Peripheral blood leukocytes from patient:		
#1–7	CML (Blast Crisis)	18–27
#8–20	CML	0.01–0.03
#21	AML	0.2
#22–28	AML	0.03–0.04
#29–33	ALL	4–10
#34–37	CLL	0.05–0.09
2. Normal pheripheral human blood lymphocytes	PHA stimulated (T Cells)	0.03–0.05
3. B-Cells		
NC 37	Normal	0.07
8392	ALL	0.1
SB	ALL	0.01
4. Cell Lines with T-Cell features		
Molt-4	ALL	26
8402	ALL	29
5. Thymus gland		
Human		18
Calf		21

* Terminal transferase assays were carried out at 37° for 1 hr in the presence of 0.1 mM Mn^{2+} in a standard reaction mixture as described in Materials and Methods.

blast phase and in 5 out of 5 patients with acute lymphocytic leukemia. Cells from four patients with chronic lymphocytic leukemia and 7 patients with acute myelogenous leukemia were negative for terminal transferase. Cells from one patient with acute myelogenous leukemia, however, showed definite but low levels of terminal transferase acitivity.

Table II: Terminal transferase in human leukemic cells

Diagnosis	Number of Cases Tested	Number Positive for TdT
1. Chronic Myelogenous Leukemia		
Acute Blast Phase	20	7
Chronic Phase	13	0
2. Acute Myelogenous Leukemia	8	1
3. Acute Lymphocytic Leukemia	5	5
4. Chronic Lymphocytic Leukemia	4	0

The distribution of terminal transferase acitivity on a sucrose gradient (5–20 %) from extracts of Molt-4 cells and cells from two CML patients in acute blast phase of the disease is shown in figure 1. The levels of activity observed in patient #1 and a T cell line (Molt 4) are similar.

Terminal transferase levels in remission: We also analyzed the cells of a CML patient whose cells had high levels of terminal transferase in the acute blast phase, after chemotherapy and induction of remission. Cells were obtained from this patient when he was in complete hematological remission and examined for the presence of terminal transferase. As shown in Table 3, the level of terminal transferase returned to the negligible levels characteristic of normal blood lymphocytes and B cell lines.

Table III: Terminal transferase in CML cells*

Diagnosis	Incorporated of [^{3}H]dGMP (nmoles per hr per 10^8 cells)
Chronic Myelogenous Leukemia	
1. Acute Blast Phase	27
2. Remission	0.1

* Terminal transferase assays were carried out at 37° for 1 hr as described in Materials and Methods.

Properties of purified terminal transferase: Terminal transferase was purified from CML cells (patient #1) by successive chromatography on DEAE cellulose, phosphocellulose and hydroxyapatite columns (5, 6, 8). Terminal transferase and DNA polymerase β were eluted together from DEAE cellulose in 0.05 M KCI

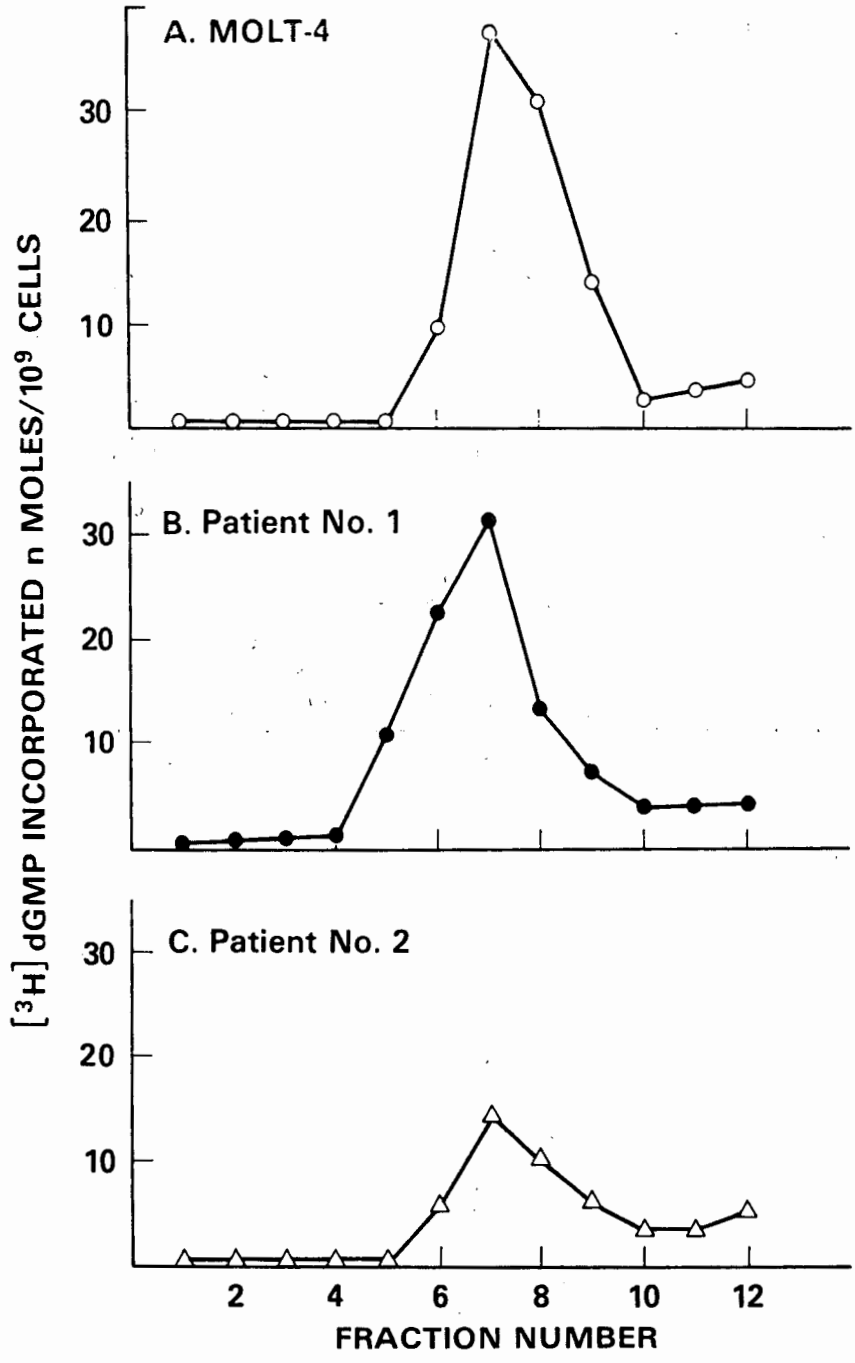

Fig. 1: *Sucrose gradient analysis of terminal transferase:* An aliquot (0.2 ml) of the cell extract was layered on top of a 4.5 ml sucrose gradient (5–20 %) made in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.5 M KCl and 0.1 mg/ml bovine serum albumin and centrifuged for 16 hrs. at 189,000 xg in a spinco SW 50.1 rotor. Fractions were collected from the bottom of the tube and an aliquot (10 µl) was assayed for terminal transferase as described in *Materials and Methods.* (A) Molt-4, ○—○; (B) Patient #1, ●—●; (C) Patient #2, △—△.

wash (5, 6, 8). The DEAE cellulose pool was subsequently chromatographed on a phosphocellulose column. Figure 2A shows the elution of terminal transferase around 0.2M KCl, whereas DNA polymerase β is eluted at 0.34 M KCl. A second minor peak of terminal transferase observed around 0.25 M KCl is a column artifact, and it is produced by the sudden increase in the salt concentration

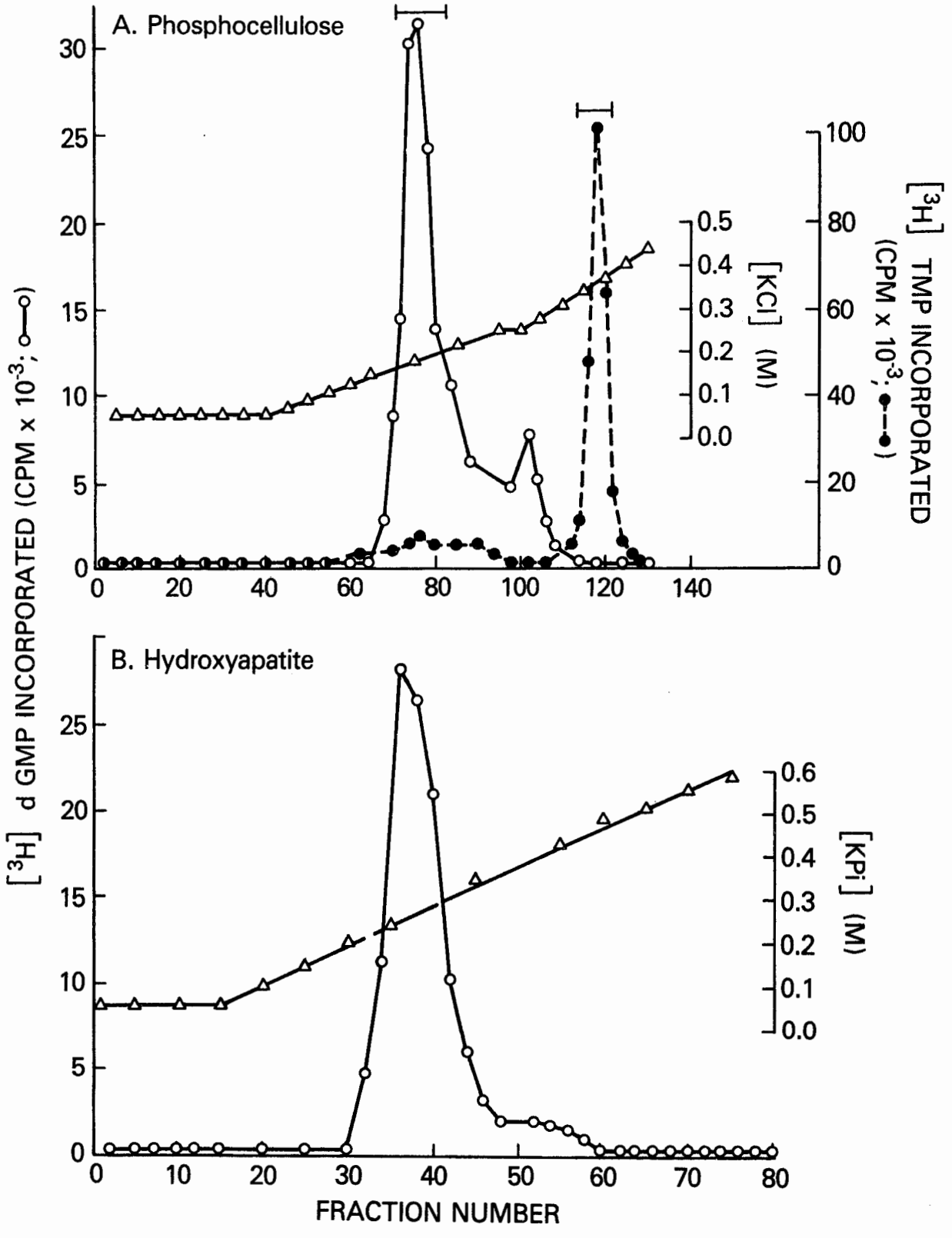

Fig. 2: A. *Chromatography of terminal transferase on phosphocellulose:* The DEAE cellulose (0.05 M KCl wash) pool containing the terminal transferase activity was adsorbed on a phosphocellulose column (Whatman P-11) (4 x 1.4 cm) equilibrated with buffer C. After washing the column with buffer C (50 ml), the column was developed with 50 ml linear gradient of KCl between 0.05 and 0.7 M. Fractions (0.5 ml) were collected and assayed for terminal transferase as described in *Materials and Methods.* DNA polymerase

assays to detect DNA polymerase β in the column fractions were carried out in a standard reaction mixture which contained $(dT)_{15}$·poly (dA) as the template-primer and [^{3}H]dTTP as the labelled substrate under conditions described in *Materials and Methods.* Poly(dA), (○—○); dT_{15}·poly(dA), (●—●).

B. *Chromatography of terminal transferase on hydroxyapatite:* The phosphocellulose fractions containing the terminal transferase activity were pooled and dialyzed before adsorption on a hydroxyapatite column (4 x 1.4 cm) equilibrated with buffer D. The column was washed with 20 ml buffer D, and then developed with 50 ml linear gradient of buffer D and 0.8 M phosphate buffer (pH 7.5) containing 1 mM DTT, 0.05 % triton X-100 and 20 % glycerol. Fractions (0.5 ml) were collected and assayed for terminal transferase as described in Materials and Methods with poly(dA) as the initiator (○—○).

of the gradient. The enzyme from the phosphocellulose column was further purified by chromatography on a hydroxapatite column. It was eluted from this column with 0.25 M phosphate buffer (figure 2B). The enzyme at this step was approximately 1000 fold purified and was used for the experiments described below.

The effect of the addition of unlabeled deoxyribonucleoside triphosphates on the activity of terminal transferase in the presence of Mn^{2+} is summarized in table 4. As shown in this table, the polymerization of one deoxyribonucleoside triphosphate is affected by the addition of other deoxyribonucleoside triphosphates, a property characteristic of terminal transferase. DNA polymerases on the other hand require all four deoxyribonucleoside triphosphates for optimum DNA synthesis.

Table IV: Effect of the Addition of Deoxyribonucleoside Triphosphates on the Polymerization of [^{3}H] dGTP Catalyzed by Terminal Deoxynucleotidyl transferase from CML cells

Substrate	[^{3}H] dGMP Incorporated per reaction*	
	pmoles	%
[H^3] dGTP	44	100
[H^3] dGTP + dATP	4.0	9
[H^3] dGTP + dATP + dCTP	2.8	6
[H^3] dGTP + dATP + dCTP + dTTP	2.0	5

* Terminal transferase assays were carried out at 37° for 30 min. in the presence of 0.1 mM Mn^{2+} in a standard reaction mixture as described in Materials and Methods. Activated salmon sperm DNA was used as the initiator at a final concentration of 50 μg/ml. 20 μM of [^{3}H] dGTP and 80 μM of the unlabeled deoxyribonucleoside triphosphates were used where indicated.

As shown in table 5 terminal transferase purified from CML cells, cell line 8402, and calf thymus gland efficiently utilizes oligo and polydeoxyribonucleotides as initiators. Oligo (dA) and poly (dA) are the most effective initiators for this enzyme whereas oligo and polyribonucleotides are very inefficient (1, 5, 6, 8).

The purified enzyme has a Mn^{2+} optimum of 0.1 mM, Mg^{2+} optimum of 7 mM (figure 3), and a pH optimum of 7.5 (figure 4). Terminal transferase activity

Table V: Comparison of response of terminal transferase from Human Leukemic cells, Calf Thymus and of the 8402 cell line to various DNA and RNA initiators

Initiator	pmoles [^{3}H] dGMP incorporated per reaction*		
	CML	Calf Thymus	8402
1. Deoxyribonucleotide			
$(dA)_{15}$	730	2500	850
$(dC)_{15}$	250	650	380
$(dT)_{15}$	80	200	180
$(dA)_n$	200	2300	730
$(dC)_n$	15	70	70
$(dT)_n$	130	400	290
2. Ribonucleotide			
$(A)_4$	5	2	9
$(A)_n$	7	2	7
$(U)_n$	3	2	4

* Terminal transferase assays were carried out at 37° for 30 min., as described in Materials and Methods, in the presence of 0.1 mM Mn^{2+}. The initiator concentration used for the assays was 50 µg/ml. $(dN)_{15}$ represents an average chain length of 15 derived from oligodeoxyribonucleotides of chain length from 12 to 18.

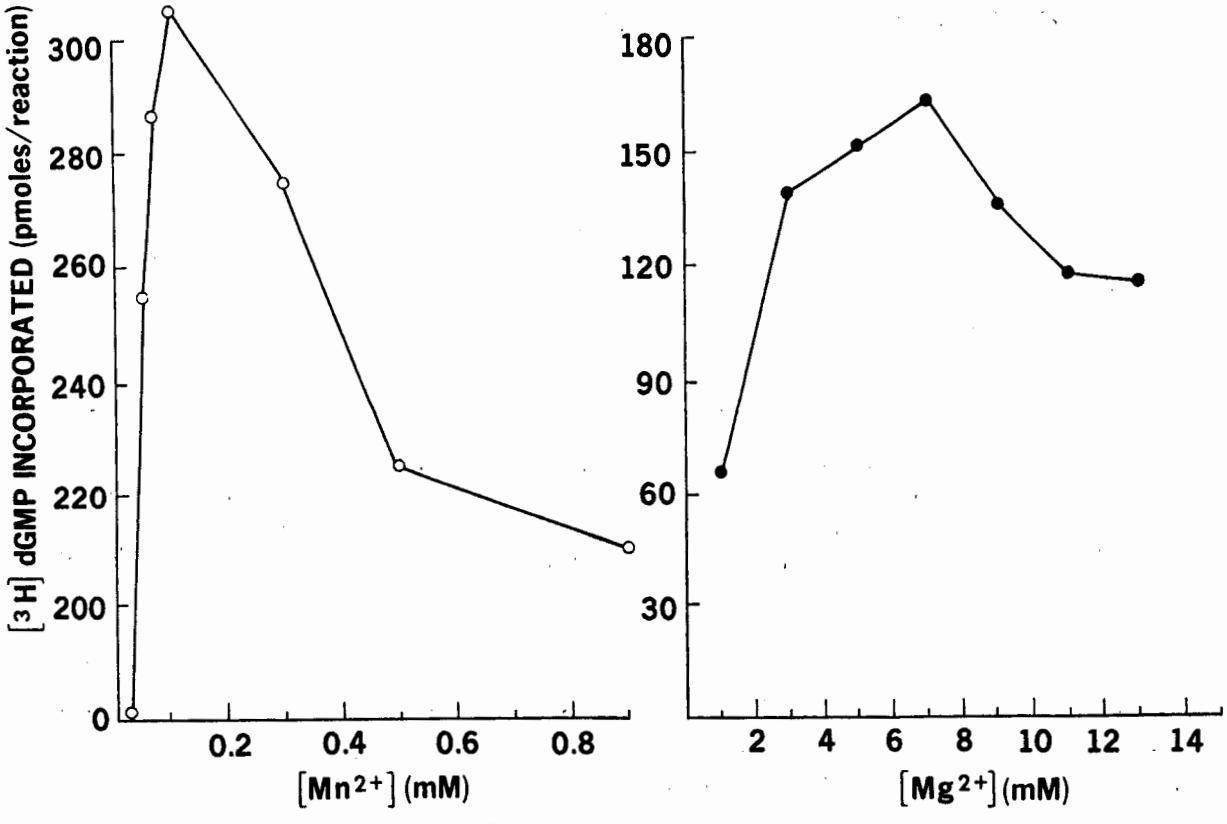

Fig. 3: Effect of divalent cation concentration on poly(dA) initiated [^{3}H]dGMP incorporation by terminal transferase. (A) Mn^{2+} (○—○); (B) Mg^{2+} (●—●).

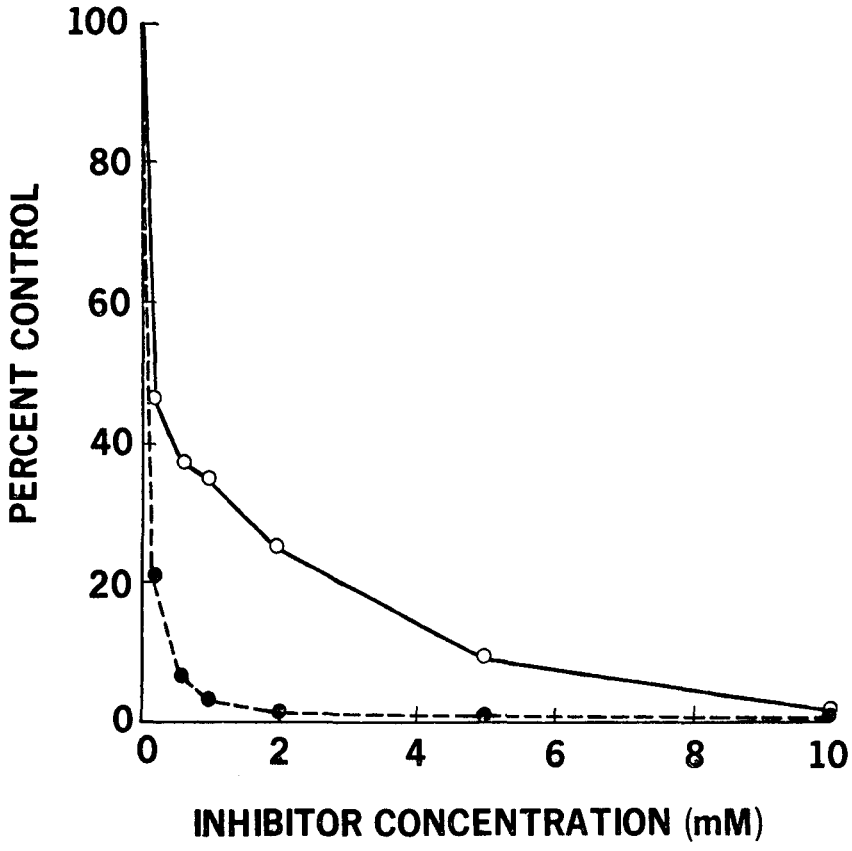

Fig. 5: Effect of inhibitor concentration on terminal transferase activity (A) N-Ethylmaleimide (○—○); (B) Sodium pyrophosphate, (●—●).

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In this report and elsewhere (5, 6) we have shown that high levels of terminal transferase are present in leukocytes of some patients with chronic myelogenous leukemia in acute blast phase of the disease. Similar results have recently been reported (7). These levels are comparable to the levels of activity observed in human and calf thymus tissue and in cell lines with T cell characteristics (Molt 4 and 8402). Insignificant levels of this activity are detected in normal cells, B cell lines, CLL and AML cells. Cells from one patient with AML, however, contained definite but low levels of terminal transferase activity. In addition we find that high levels of terminal transferase detected in the leukocytes of a CML patient in acute "blast" phase of the disease returned to levels present in normal cells after hematological remission. These results suggest that in some CML patients in acute blast phase of the disease, there is an induction of terminal transferase which returns to negligible levels (observed in normal cells) after hematological remission. It may, therefore, be possible to use terminal transferase as a sensitive biological marker to follow these patients during treatment and in remission.

In our studies so far, we have observed high levels of terminal transferase in 7 out of 20 patients with chronic myelogenous leukemia in acute blast phase of the disease, demonstrating that this enzyme is not present in all CML cells in blast phase. All of the patients with acute lymphocytic leukemia, on the other hand,

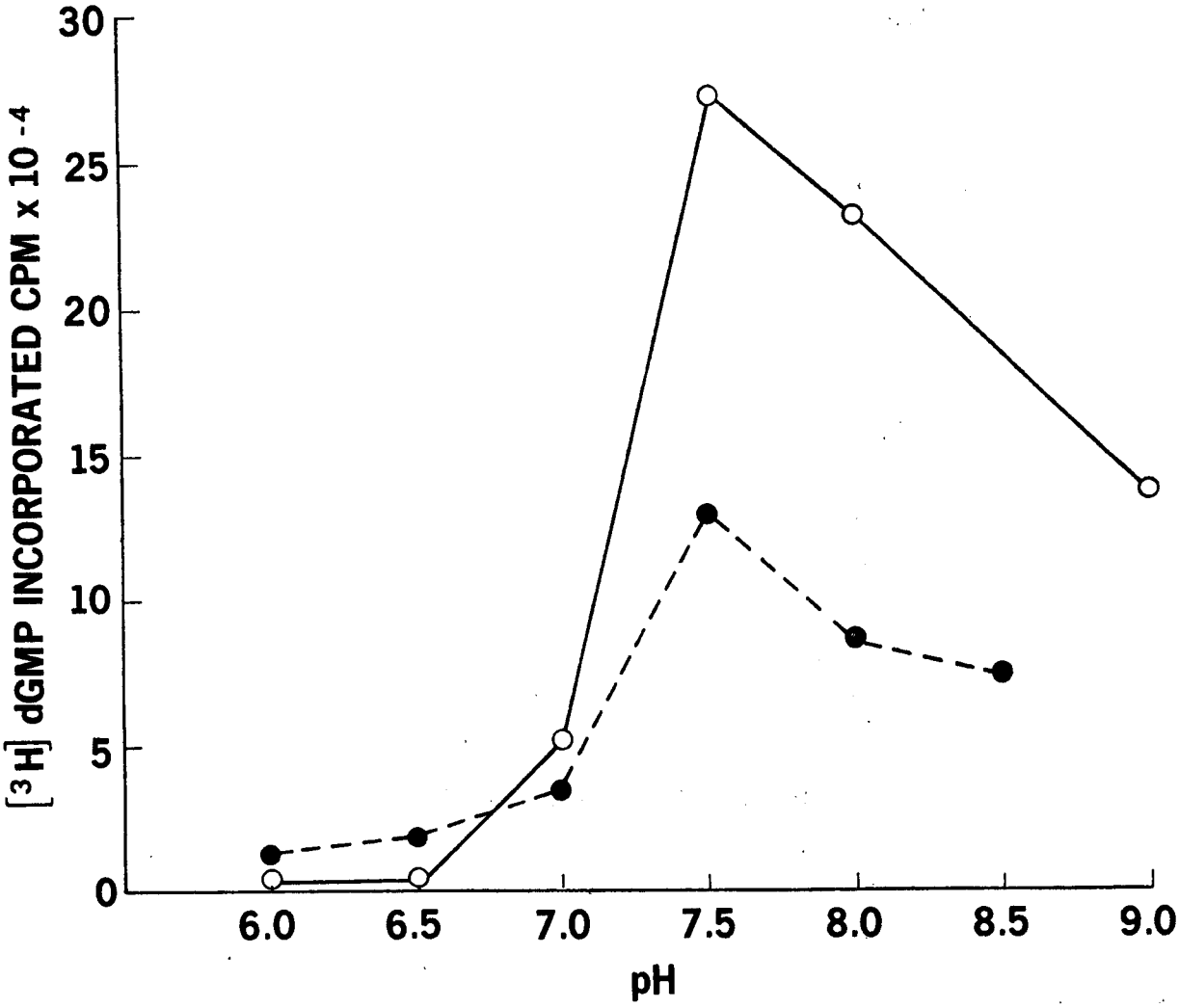
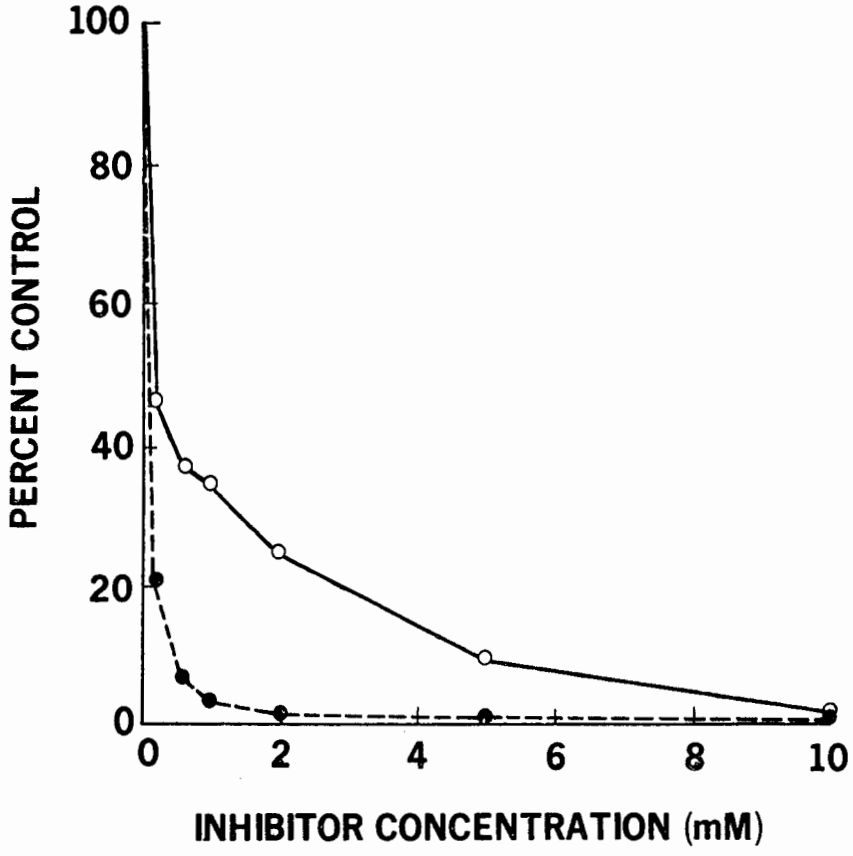


Fig. 4: Effect of pH on poly dA initiated [^{3}H]dGMP incorporation by terminal transferase. Mn^{2+} (○—○); Mg^{2+} (●—●)..

was inhibited by N-ethylmalemide and sodium pyrophosphate as shown in figure 5. DNA polymerase β, on the other hand, is only inhibited by N-ethylmalemide at high concentrations (15). The purified enzyme has a sedimentation value of 3.4S as estimated by sucrose density gradient centrifugation (16) with ovalbumin as a marker (figure 6). This value is similar to the value reported for terminal transferase from thymus gland and from cell lines with T cell characteristics (1, 5, 6, 8).

Discussion

Terminal transferase, an enzyme first isolated from calf thymus was considered to be specific for thymus tissue since it was not detected in bone marrow, liver, lungs, lymph nodes and spleen (17). Recent studies have shown that this enzyme is also present in peripheral blood from many patients with acute lymphocytic leukemia (2, 6, 7) and in some patients with acute myelomonocytic leukemia (3) or chronic myelogenous leukemia in acute "blast" phase of the disease (5, 6, 7). Low levels of terminal transferase have also been reported in normal bone marrow



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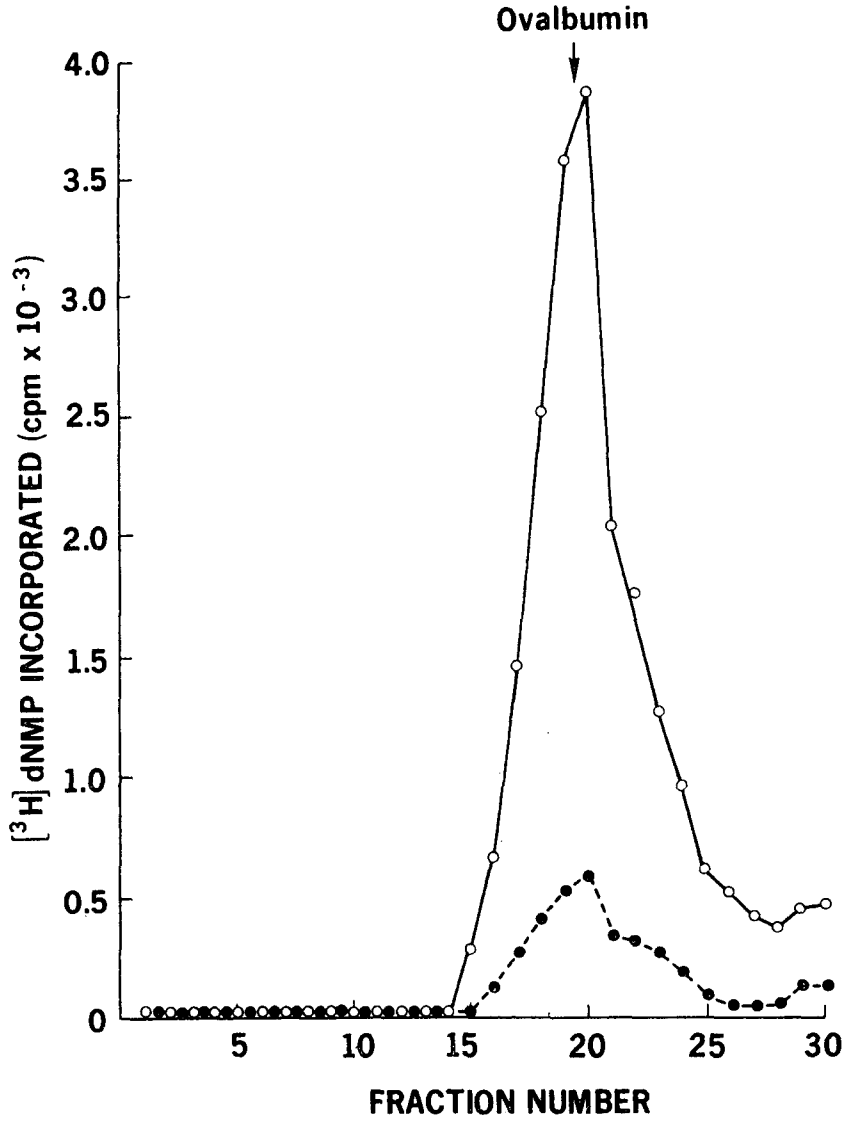

Fig. 6: *Sedimentation analysis of terminal transferase:* An aliquot (0.2 ml) of the purified enzyme was layered on top of a 4.5 ml sucrose gradient (5–20 %) made in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.5 M KCl, and centrifuged for 16 hrs. at 189,000 xg in spinco SW 50.1 rotor. Ovalbumin as a marker protein was centrifuged on a parallel gradient. Fractions (0.15 ml) were collected from the bottom of the tube and an aliquot (10 µl) was assayed for terminal transferase as described in Materials and Methods. [^{3}H]dGMP incorporation with $(dA)_{15}$ as initiator (○—○). [^{3}H]dTMP incorporation with $(dT)_{15}$·poly (dA) as initiator (●--●).

contained terminal transferase. These results point to the possibility that in some CML in acute blast phase, undifferentiated lymphoblasts (which may be precursor to T cells) rather than myeloblasts may be the cells which proliferate. The possibility of lymphoblastic conversion in CML has recently been suggested (7, 18, 19). If lymphoblastic conversion occurs in some cases of CML as it appears, then terminal transferase levels may also be useful as a diagnostic marker to predict the type of CML patients that may respond to treatment with predisone and Vincristine.

Acknowledgement

We thank Dr. Bayard Clarkson, Dr. Timothy Gee and Dr. Yashar Hirshaut, Sloan Kettering Institute; Dr. Kenneth McCredie, M. D. Anderson Hospital; Dr. Martin Oken, University of Minnesota; Dr. Bruce Chabner, National Cancer Institute; and Dr. George Canellos, Harvard Medical School for Leukemic Cells. We thank Bong Hee Ro for expert technical assistance.

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