Biochemical and Immunological Properties of Human Terminal Deoxynucleotidyl Transferase Purified from Blasts of Acute Lymphoblastic and Chronic Myelogenous Leukemia

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ABSTRACT Terminal deoxynucleotidyl transferase was purified to homogeneity from the blasts of eight patients with leukemia and compared with purified transferase from normal human and calf thymus. In two cases phenylmethanesulfonylfluoride was added during purification to reduce proteolysis. Comparative kinetic analyses of the purified enzymes indicated no differences in catalytic properties. There was substantial variation in the molecular structure of terminal transferase on denaturing polyacrylamide gels: (*a*) a protein that migrated as a single polypeptide with M_r = 62,000 was isolated from two patients with acute lymphoblastic leukemia and from MOLT-4 cells; (*b*) a protein that migrated as a single polypeptide with M_r = 42,500 was isolated from two patients with acute lymphoblastic leukemia; (*c*) a protein that migrated as a single polypeptide with M_r = 42,500 was isolated from two patients with chronic myelogenous leukemia in blast crisis; (*d*) a protein that migrated as two nonidentical subunits of M_r = 27,000 and 10,000, respectively, was isolated from two additional patients with chronic myelogenous leukemia in blast crisis. The subunit structure of *d* is characteristic of the homogeneous enzymes purified from human and calf thymus. Neutralizing and precipitating antibodies to terminal transferase from human lymphoblasts and calf thymus have been produced in rabbits and goats. Antisera directed against either human or calf antigens neutralize enzymatic activity and precipitate all forms of human terminal transferase. The multiple human forms give reactions of antigenic identity by immunodiffusion, but differ antigenically from the calf enzyme. The multiple forms of terminal transferase could represent physiological processing, artifactual degradation, or isozymes coded by several genes.

INTRODUCTION

Terminal deoxyribonucleotidyl transferase (terminal transferase, EC, 2.7.7.31)[1] is a DNA-polymerizing enzyme, which is widely used as a marker of neoplastic cells in certain human diseases (1–4). While an in vivo function has not yet been established for terminal transferase, a role in immunological programming has been suggested as a result of its location in mammalian thymus and bone marrow (5, 6). A cytoplasmic location of thymic terminal transferase was established by subcellular fractionation (7) and by immunofluorescence detection of the enzyme in small thymocytes from adult animals (8). On the other hand, terminal transferase in bone marrow cells, in immature thymocytes (9), and in leukemic lymphoblasts (10) is present in the nucleus, as demonstrated by immunofluorescence. As a consequence, it has been suggested that terminal transferase in lymphoid tissue undergoes posttranslational cleavage and may form molecules that are enzy-

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[1] *Abbreviations used in this paper*: ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; $p(dA)_{\overline{50}}$, poly-(deoxyadenylic acid) with an average chain length of 50 residues; PMSF, phenylmethane sulfonylfluoride; terminal transferase, terminal deoxyribonucleotidyl transferase.

matically active in vitro, but not biologically active in vivo (9). Recently, we have reported a high molecular weight form of terminal transferase in blasts of a patient with acute lymphoblastic leukemia (ALL) (11). The structural characteristics of this high molecular weight enzyme are different from the enzyme purified to homogeneity from calf thymus (12). The differences between human lymphoblasts and calf thymus in subcellular location and structure of terminal transferase stimulated us to examine whether these differences represent a species or tissue variation or are related to the type of leukemia. We have purified and characterized terminal transferase from normal human thymus and from leukemic cells of patients with ALL and chronic myelogenous leukemia in blast crisis.

METHODS

Chemicals. Radioactive deoxynucleoside 5'-triphosphates were obtained from New England Nuclear, Boston, Mass. All nonradioactive nucleosides and nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo.), and were further purified (13). Phenylmethanesulfonylfluoride (PMSF) was a product of Sigma Chemical Co. Oligo(dT)cellulose (type 7) was obtained from P-L Biochemicals, Inc., (Milwaukee, Wis.). Bio-Gel HTP (hydroxylapatite), Biolytes (ampholytes), pH 3–10, and pH 8–10, Affigel Blue (Cibacron Blue F3GA-Sepharose) and Chelex-100 were purchased from Bio-Rad Laboratories, (Richmond, Calif.). CPG/460 glycophase G was the product of Pierce Chemical Co., (Rockford, Ill.). Goat anti-rabbit and rabbit anti-goat IgG were obtained from N. L. Cappell Laboratories, Inc. (Cochranville, Pa.). Double diffusion plates (Ouchterlony plates) were purchased from Miles Laboratories Inc. (Elkhart, Ind.). All ultrafiltration experiments were conducted using PM-10 membranes obtained from Amicon Corp. (Lexington, Mass.). All other chemicals were reagent grade from commercial sources.

Enzyme assays. The assay of human terminal transferase has been described (14). The components of the assay in a total vol of 125 μl were: 0.2 M potassium cacodylate, pH 7.5, 1 mM 2-mercaptoethanol, 0.010 mM poly(deoxyadenylic acid) with an average chain length of 50 residues [$p(dA)_{50}$], and 1 mM [^{3}H] deoxyATP (dATP) (100 cpm/pmol) with 1.5 mM $MgCl_2$, or 1 mM [^{3}H]deoxyguanosine triphosphate (dGTP) (100 cpm/pmol) with 0.5 mM $MnCl_2$. For the assay of the calf thymus enzyme, conditions were identical except that the polymerization of deoxyguanosine 5'-triphosphate onto $p(dA)_{\overline{50}}$ was conducted in the presence of 8 mM $MgCl_2$. The reactions were incubated at 35°C for varying time periods, and terminated by application of 25-μl aliquots onto GF/C glass fiber papers as described (15). 1 U of enzyme activity is defined as 1 nmol of radioactive deoxynucleotide incorporated per hour, and specific activity is expressed as units of activity per milligram of protein.

Source of leukemic cells. Human leukemic blasts were collected from patients undergoing therapeutic leukapheresis (16). This procedure normally yields between 200 and 1,000 g of cells per patient. The lymphoblasts were purified by dextran sedimentation (2). MOLT-4 is a terminal transferase containing lymphoblastoid cell line originally established from a patient with T-marked acute lymphoblastic leukemia (17). At the time of enzyme purification, our line of MOLT-4 was no longer T marked. Variation in the surface markers of MOLT-4 has been noted by numerous investigators. In one case, a spleen was obtained from a patient with ALL in relapse who required splenectomy. The material was immediately frozen and stored at −70°C.

Purification of terminal transferase from human blasts. Preparations of terminal transferase from leukemic blasts were purified using the procedure summarized in Table I. In two preparations, the protease inhibitor, PMSF, was added in the extraction buffer at a concentration of 1 mM. A suspension of packed lymphoblasts (up to 2 kg of cells) was diluted with 3 vol of extraction buffer (0.25 M potassium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol) and mixed for 1 h at 4°C. Our best results for extraction were obtained by passing the semithawed tissue through a chilled meat grinder before addition of the extraction buffer. The suspension was centrifuged at 10,000 *g* for 1 h at 4°C to yield the crude extract supernate (fraction I).

Fraction I (supernate) was diluted with 3 vol of 1 mM 2-mercaptoethanol and added to a carboy containing 2 liters of phosphocellulose slurry, equilibrated with 50 mM potassium phosphate, pH 7.2, 1 mM 2-mercaptoethanol (buffer A), per kilogram of tissue. The mixture was stirred for 2 h at 4°C, and the supernatant decanted after settling of the phosphocellulose. The gel was washed with equilibration buffer, and finally with 75 mM potassium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol. To facilitate elution of the bound terminal transferase, the phosphocellulose was poured into a glass column (10-cm Diam) and the enzyme was eluted using 0.3 M potassium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol, to yield fraction II. This fraction was pooled and concentrated by precipitation with 75% saturated ammonium sulfate. After centrifugation at 15,000 *g* the precipitated pellets were redissolved in buffer A.

TABLE I
Purification of Terminal Deoxynucleotidyl Transferase from 1 kg of Human Leukemic Lymphoblasts

	Fraction	Volume	Protein	Specific activity	Total activity	Yield	Purification
		ml	*mg*	*U/mg*	*U*	*%*	*-fold*
I	Crude extract	4,200	74,800	10	773,940	100	1
II	Phosphocellulose	500	7,250	108	789,474	102	10
III	Hydroxylapatite-I	200	3,000	244	734,200	94	23
IV	Affigel blue	12	156	3,353	523,112	67	325
V	Hydroxylapatite-II	8	80	7,121	569,671	73	691
VI	Oligo(dT)cellulose	6.5	5.2	111,517	579,889	74	10,826

Fraction II was diluted with 3 vol of 1 mM 2-mercaptoethanol and applied to a column of hydroxylapatite consisting of 300 ml bed vol/kg tissue originally prepared. The column was washed with several column volumes of equilibration buffer (buffer A) and the terminal transferase was eluted with 0.3 M potassium phosphate, pH 7.2, 1 mM 2-mercaptoethanol. The fractions with terminal transferase activity were pooled and concentrated by ammonium sulfate. The precipitate was redissolved in buffer A. (fraction III).

Fraction III was diluted with 5 vol of 1 mM 2-mercaptoethanol, and applied to a column of Affigel Blue consisting of 1 liter bed vol/kg of original tissue extracted. After exhaustive washing with buffer A, terminal transferase activity was eluted by 0.3 M potassium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol. These fractions were pooled and concentrated by ultrafiltration to yield fraction IV.

Fraction IV was diluted with 5 vol of 1 mM 2-mercaptoethanol and applied to a column of hydroxylapatite (50 ml bed vol/kg of tissue) equilibrated with buffer A. After washing with several column volumes of buffer A, the column was developed using a linear gradient from 50 to 350 mM potassium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol (total gradient volume was 500 ml/kg of tissue). Fractions containing terminal transferase activity were pooled and concentrated by ultrafiltration to yield fraction V.

Fraction V was diluted with 5 vol of 1 mM 2-mercaptoethanol (containing 10 μM $MgCl_2$) and applied to a column of oligo(dT)cellulose having the dimensions of 2.5 × 5.0 cm for all sample sizes. The column was washed with buffer A. Terminal transferase activity was eluted using a linear gradient (0–1.5 M KCl) in buffer A. A gradient vol of 350 ml was used. Fractions containing terminal transferase activity were pooled and concentrated by ultrafiltration.

Purification of terminal transferase from human thymus. Terminal transferase protein was purified to homogeneity by a modification of the procedure described above for human blasts. Because of the limited availability of fresh human thymus, we designed a shortened procedure. This method relies specifically on affinity chromatography procedures, and can be used only with small amounts of tissue (up to 50 g). Minced human thymus obtained during thoracic surgery (~15 g) was added to 40 ml of 0.25 M potassium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol, and sonicated (three 15-s bursts). The resulting suspension was stirred at 4°C for 60 min, and centrifuged at 25,000 *g* for 30 min. The supernate obtained represented fraction I (Table II).

Fraction I was diluted with 4 vol of 1 mM 2-mercaptoethanol and 0.1 mM $MgCl_2$, and applied to a column of oligo(dT)-cellulose (2.5 × 5.0 cm) equilibrated with buffer A. After a wash with 100 ml of buffer A, terminal transferase activity was eluted with a linear gradient (300 ml) from 0 to 1.2 M potassium chloride (in buffer A). Fractions containing terminal transferase activity were pooled and concentrated by ultrafiltration to yield fraction II.

Fraction II was diluted with 5 vol of 1 mM 2-mercaptoethanol containing 0.1 mM $MgCl_2$, and applied to a column (1.2 × 5.5 cm) of oligo$(dT)_{6-15}$ $(dA)_{50-100}$ cellulose. This column material was prepared in our laboratory by polymerization of dATP to an average chain length of 50–100 residues onto oligo(dT)cellulose using calf thymus terminal transferase. After a wash with 75 ml of buffer A, terminal transferase activity was eluted with a linear gradient (200 ml) from 0 to 1.2 M potassium chloride in buffer A. Fractions containing terminal transferase activity were pooled and concentrated to yield fraction III.

Fraction III was diluted with 7 vol of 25 mM potassium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol, and applied to a column of hydroxylapatite (1.2 × 2.5 cm). After a wash with 50 ml of buffer A, terminal transferase activity was eluted with a linear gradient (200 ml) from 50 to 350 mM potassium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol. Fractions containing terminal transferase activity were pooled and concentrated to yield fraction IV. The purified protein which we obtained (0.11 mg) had a final specific activity of 55,180 U/mg, representing an overall yield of 67% (Table II). Because of instability inherent in very diluted samples of terminal transferase, this specific activity represents a minimum estimated value. Stabilization of the enzyme activity by glycerol or exogenously added solutions of bovine serum albumin was ineffective.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The subunit structure of homogeneous preparations of human terminal transferase was analyzed by denaturing gel electrophoresis in the presence of SDS. This procedure was conducted according to the methods of Laemmli (18) or Weber and Osborn (19) using a slab gel apparatus: either a model 4214 cell and model 4200 tank, manufactured by Ortec, Inc. EG & G, Inc., (Oak Ridge, Tenn.), or a model 220 cell obtained from Bio-Rad Laboratories. A pulsed, constant power supply (Ortec, Inc., model 4100) was used for all experiments. Protein samples, containing 10 mM 2-mercaptoethanol, 25% glycerol, 2% SDS, 0.2% bromphenol blue, and either 0.125 M Tris-HCl, pH 6.8 for the Laemmli system, or the sodium phosphate buffer components of the Weber and Osborn gel system, were immersed in a boiling water bath for 5 min. A set of protein standards (bovine serum albumin, relative mol wt (M_r) = 67,000; ovalbumin, M_r = 45,000; carbonic anhydrase, M_r = 30,000; and cytochrome *c*, M_r = 11,500) were run simultaneously in separate gel tracks. In several instances, additional molecular weight markers were also employed (lactate dehydrogenase, M_r = 35,000; α-chymotrypsinogen A, M_r = 26,000; and lysozyme, M_r = 14,300). Gels were stained by incubation for 12 h in a solution of 0.2% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, and destained in a solution containing 50% methanol and 10% acetic acid.

Isoelectric focusing. The procedure for analytical slab gel isoelectric focusing of human terminal transferase has been

TABLE II

Purification of Terminal Deoxynucleotidyl Transferase from Human Thymus

	Fraction	Volume	Protein	Specific activity	Total activity	Yield	Purification factor
		ml	*mg*	*U/mg*	*U*	%	*-fold*
I	Crude extract	52	161.2	55.8	9,000	100	1.0
II	Oligo dT cellulose	5	1.75	3,900	6,825	75	69.9
III	Oligo dA cellulose	1.2	0.24	29,315	7,035	78	525.3
IV	Hydroxylapatite	0.1	0.11	55,180	6,070	67	988.9

described in detail elsewhere (11). In general, 5–10 μg of homogeneous terminal transferase protein was analyzed in an ampholyte system consisting of 2% pH 3–10 Biolytes and 0.5% pH 8–10 Biolytes. Electrophoresis of these samples was conducted in a 6% polyacrylamide gel, using a pulsed, constant power supply.

Enzyme kinetics. Michaelis constants and maximum velocities were calculated by the least squares method of Cleland (20) using a Wang 2200 programmable computer (Wang Laboratories, Inc., Lowell, Mass.). Several of these determinations have been published (11, 21).

Antisera. Antisera were raised to terminal transferase purified from human leukemic cells and calf thymus (11, 12). Rabbits were initially immunized with three injections, 50 μg each, of glutaraldehyde cross-linked terminal transferase using the schedule and conditions developed by Bollum (22). Responders (six of six rabbits) were injected subcutaneously with 50 μg of calf or human soluble native enzymes, as appropriate, and bled at 7 and 9 d from the ear vein. Intravenous injections of 10 μg calf or 20 μg human soluble enzyme were continued at 1-mo intervals. Goats were injected using a similar schedule, but at five times the dose of antigen.

Serum was collected, heat inactivated at 56°C for 30 min, and passed over a 2 × 4.5-cm Chelex-100 column equilibrated with phosphate-buffered saline. Certain antisera were purified by passage over a CPG/460 glycophase G column to which 750 μg of homogeneous calf thymus terminal transferase was coupled (9). The purified antibody was concentrated by ultrafiltration, adjusted to 20 mg/ml in phosphate-buffered saline, and stored in 0.1 ml aliquots at −70°C.

Antibody detection. Microdiffusion analyses were carried out on double immunodiffusion plates. Sera were tested against 50–500 ng of terminal transferase in a solution containing 5 mg/ml bovine serum albumin (21). To test inhibition of enzyme activity, serum was diluted serially with 5 mg/ml bovine serum albumin in phosphate-buffered saline. Extracts of calf thymus, human thymus, and leukemic blasts representative of each of the molecular types of terminal transferase were prepared and were diluted with 5 mg/ml bovine serum albumin in phosphate-buffered saline to contain 100 U terminal transferase/ml (23). Equal amounts (10 μl) of diluted serum and tissue extract were mixed and incubated for 15 h at 4°C. The antigen-antibody mixture was tested directly for terminal transferase activity. Control dilutions of normal rabbit serum were included in each titration. A comparison of the neutralization of each fraction of enzyme by serum was made by constructing plots of terminal transferase relative activity (%) vs. the log of the quantity of serum protein.

Detection of antigen by immunofluorescence. The cut surface of a human thymus was touched to a glass slide to prepare cells suitable for immunocytochemistry. The first stage consisted of 15 μl of appropriately diluted serum or purified antibody (4, 10, 22, 24, 25). The second stage consisted of 15 μl of 250 μg/ml fluorescein conjugated $F(ab')_2$ goat antirabbit or rabbit anti-goat IgG. The percentage of fluorescent cells and the intracellular distribution of the fluorescence were determined using a fluorescence microscope with incident light excitation.

RESULTS

An analysis of the biochemical and biophysical properties of human terminal transferase required that the enzyme be purified to homogeneity from several different sources. Our criteria for homogeneity are: (*a*) the presence of a single polypeptide on SDS polyacrylamide gels or the presence of the previously reported two-subunit composition observed for the calf thymus enzyme (12); (*b*) a single band on nondenaturing isoelectric focusing gels (examples shown in Fig. 1); and (*c*) a specific activity that corresponds to protein preparations that were defined as homogeneous according to the criteria of nondenaturing polyacrylamide gel electrophoresis, SDS polyacrylamide gel electrophoresis, and analytical ultracentrifugation (12). The enzymes were purified from acute leukemic lymphoblasts and from the blasts of patients with chronic myelogenous leukemia (CML) in blast crisis (11). Terminal transferase isolated from greater than 100 g of starting material exhibited specific activities (~100,000 U/mg) comparable to the specific activity obtained for homogeneous calf thymus terminal transferase (12). Our standard purification procedure, designed to circumvent the low pH step and extended time required for purification (12), was less successful for the isolation of terminal transferase from small quantities of tissue (<100 g). Thus, the specific activities of preparations 1 and 3 (Table III) represent lower limits of specific activity generally seen with homogeneous enzyme, due to the lack of stability inherent in very diluted samples of terminal transferase. To purify the enzyme from very small quantities of human thymus (<20 g), a shorter but highly successful purification method was devised (Table II). When terminal transferase was puri-

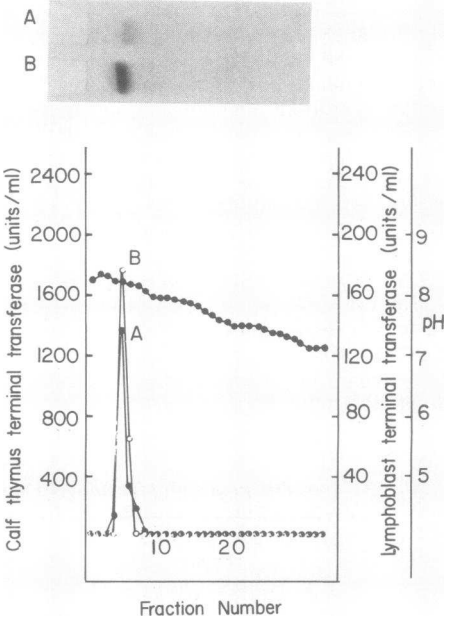

FIGURE 1 Analytical nondenaturing isoelectric focusing polyacrylamide gel electrophoresis of human (A) and calf thymus (B) terminal transferase. The electrophoresis is described in Methods. The activities corresponding to each protein band are shown in the accompanying figure.

TABLE III

Physical Characteristics of Terminal Deoxynucleotidyl Transferase Purified from Several Sources

Preparation No.	Tissue* description	Intracellular‡ localization	Original cell weight	Amount of purified enzyme	Specific activity	Subunit structure	Turnover number	pI	Michaelis constants K_m (app) dATP	Michaelis constants K_m (app) $p(dA)_{50}$
			g	*mg*	*U/mg*	M_r	*mol/h/mol protein* $\times 10^{-3}$		*mM*	μM
1	ALL blasts§	Nuclear	75	0.3	47,085	62,000	3.0	ND	ND	ND
2	ALL blasts§	Nuclear	300	0.3	82,160	62,000	5.1	8.2	0.2	0.6
3	MOLT-4	Nuclear	40	0.1	55,550	62,000	3.4	8.2	ND	ND
4	ALL blasts‖	ND	1,000	5.2	111,517	42,500	4.7	8.2	0.1	ND
5	ALL blasts§	ND	500	0.6	95,100	42,500	4.0	ND	ND	ND
6	CML blasts¶	Nuclear and cytoplasmic	165	3.7	121,725	42,500	5.1	8.2	0.1	0.4
7**	CML blasts¶	Nuclear and cytoplasmic	132	0.8	131,760	42,500	5.6	8.2	ND	ND
8	CML blasts¶	Nuclear and cytoplasmic	120	0.3	105,555	27,000 and 10,000	3.9	ND	ND	ND
9	CML blasts¶	ND	807	0.8	121,095	27,000 and 10,000	4.5	8.2	0.1	0.4
10	Human thymus	Nuclear and cytoplasmic	15	0.1	55,180	27,000 and 10,000	2.0	ND	ND	ND
11	Calf thymus	ND	2,500	2.1	111,205	30,500 and 9,500	4.5	8.2	0.1	0.6

ND, not determined.

* Preparations 1, 2, and 3 were characterized as ALL, non-B and non-T marked; a MOLT-4 clone grown in our laboratory was not T marked at the time of enzyme purification; cells from preparation 7 formed fewer than 1% E rosettes; preparation 8 contained 92% blasts, 22% of which formed E rosettes (this group of cells were classified as T marked); preparations 4, 5, 6, and 9 were not typed for surface markers.

‡ The intracellular localization was determined by immunofluorescence.

§ ALL blasts were obtained from patients with adult ALL.

‖ Cells were obtained from the blast-infiltrated spleen of a patient with ALL.

¶ CML blasts were isolated from patients with chronic myelogenous leukemia in blast crisis, where cells were found to contain terminal transferase.

** 1 mM PMSF, a protease inhibitor, was added during the initial stages of purification.

fied from 15 g of human thymus by the standard procedure using PMSF, only trace amounts of protein were available for characterization, while the modified procedure (Table II) yielded over 0.1 mg of purified protein. The enzymes with specific activities of <80,000 (preparations 1, 3, and 10) conformed to our other criteria for homogeneity. Table III summarizes characteristics of terminal transferase from calf thymus, human thymus, ALL blasts and CML blasts. The kinetic properties of the purified enzymes from acute lymphoblastic leukemia (11), human thymus, and blasts of patients with CML in crisis (21) were similar. None of the enzymes differed kinetically from terminal transferase purified from calf thymus (12, 26).

Blasts used in preparations 1 and 2 were null (non-B and non-T marked). Cells used in preparation 3 (MOLT-4) originally were established from a leukemic conversion of lymphoma and formed E rosettes (17). The clone grown in our laboratory formed <1% E rosettes. Blasts used in preparations 4 and 5 were not typed for surface markers; preparations 6, 7, and 8 were prepared from cells of patients in crisis at the time of analysis, but whose cells had contained the Philadelphia chromosome when in stable phase. Preparation

6 was not typed for surface markers. Blasts used in preparation 7 formed <1% E rosettes. Preparation 8 contained 92% blasts, 22% of which formed E rosettes, and the leukemia was classified at T-marked. Cells used in preparation 9 were not typed for surface markers.

The intracellular location of terminal transferase was readily determined by immunocytochemical detection of terminal transferase antigen with antiserum. In all preparations of ALL and MOLT-4 cells, terminal transferase was localized by immunofluorescence in the nucleus. When cells from patients with CML in blast crisis were tested, terminal transferase antigen was found in both the nucleus and cytoplasm of most cells. Human thymocytes appear to have granulated nuclear and cytoplasmic terminal transferase which is not as diffuse as seen in CML cells. Patterns of immunofluorescence of thymocytes, ALL blasts and CML blasts were quite distinctly and reproducibly different (27).

At least three structural forms of terminal transferase have been found (Fig. 2). Structural determinations were made over a 2-yr time span after each new enzyme purification. The gel preparations and procedures vary slightly and are detailed in the figure legend. The subunit structure of the purified enzyme from two patients with null cell ALL, and from MOLT-4 cells, is observed as a single polypeptide having a M_r = 62,000 as shown in Fig. 2a and b. In two additional enzyme preparations from untyped ALL, we found a single polypeptide species with a M_r = 42,500 (Fig. 2c). Two types of subunit structure have been observed in enzyme from four patients with CML in blast crisis: (*a*) from two patients we isolated terminal transferase with a subunit mol wt of 42,500 (Fig. 2d); one of these patients' cells was not typed for surface markers, while the other formed fewer than 1% E rosettes; (*b*) from two patients we isolated terminal transferase with two nonidentical subunits of M_r = 27,000 and 10,000, (Fig. 2f and g). The cells of one of these patients were T marked. Cells from the other patient were not typed. The denatured subunit structures of enzyme isolated from either calf thymus or human thymus were comparable to the structure of the enzyme from two patients with CML in blast crisis (M_r = 27,000 and 10,000, for human thymus, Fig. 2h; and M_r = 30,500 and 9,500, for calf thymus, Fig. 2i).

As one test for possible proteolysis, a potent inhibitor of a group of serine proteases, PMSF, was added during the initial stages of enzyme purification. The cells utilized for this purification were obtained from a patient with CML in blast crisis. Using the procedure in Table I we obtained a protein (M_r = 42,500) that migrated as a single polypeptide on both denaturing polyacrylamide gels and on nondenaturing polyacrylamide isoelectric focusing gels. A protein with a mol wt of 62,000 was not observed. We do not know if all proteases that may be present in the extracts of this cell preparation are inhibited by PMSF. We are therefore not able to rule out proteolysis by PMSF-insensitive proteases.

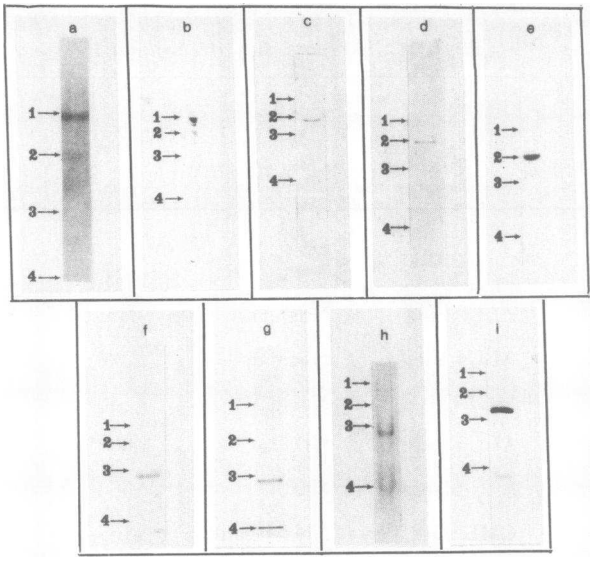

FIGURE 2 SDS-polyacrylamide gel electrophoresis of purified preparations of terminal transferase from the blasts of patients with ALL and CML in blast crisis, from normal human thymus, and from calf thymus. Preparations are listed in Table III. The electrophoresis of each sample is described in Methods. Sample concentrations varied from 5 to 15 μg. Standards used for molecular weight standardization were: (1) bovine serum albumin (M_r = 67,000); (2) ovalbumin (M_r = 45,000); (3) carbonic anhydrase (M_r = 30,000); (4) cytochrome *c* (M_r = 11,550). A description of each of the gels is as follows: (*a*) 6% stacking and 12% separating polyacrylamide gel according to the method of Laemmli (18) of terminal transferase purified from the blasts of a patient with ALL (preparation 2). (*b*) 10% running polyacrylamide gel according to the method of Weber and Osborn (19) of terminal transferase purified from the blasts of a patient with ALL (preparation 1). (*c*) 6% stacking and 12% separating polyacrylamide gel according to the method of Laemmli (18) of terminal transferase purified from the blast-infiltrated spleen of a patient with ALL (Preparation 4). (*d*) 10% running polyacrylamide gel according to the method of Weber and Osborn (19) of terminal transferase purified from the blasts of a patient with CML in blast crisis (preparation 6). (*e*) 8% running polyacrylamide gel according to the method of Weber and Osborn (19) of terminal transferase purified from the blasts of a patient with ALL (preparation 5). (*f*) 6% stacking and 12% separating polyacrylamide gel according to the method of Laemmli (18) of terminal transferase purified from the blasts of a patient with CML in blast crisis (preparation 9). (*g*) 6% stacking and 12% separating polyacrylamide gel according to the method of Laemmli (18) of terminal transferase purified from the blasts of a patient with CML in blast crisis (preparation 8). (*h*) 12% running polyacrylamide gel according to the method of Weber and Osborn (19) of terminal transferase purified from normal human thymus (preparation 10). (*i*) 6% stacking and 12% separating polyacrylamide gel according to the method of Laemmli (18) of terminal transferase purified from calf thymus (preparation 11).

When data are summarized (Table III), ALL blasts consistently have either the M_r = 62,000 or 42,500 sub-

unit species. A M_r of 42,500 polypeptide or a M_r = 27,000 and 10,000 subunit pattern is associated with CML blasts. The low molecular weight enzyme with two nonidentical subunits (M_r = 27,000/10,000 in man) is observed in human and calf thymus. We have attempted to correlate molecular weight of terminal transferase protein with its intracellular localization by immunofluorescence techniques. Considering only the preparations listed in Table III, we find that the cells (non-T and non-B) from which the M_r = 62,000 form of the enzyme was isolated (preparations 1, 2, and 3) give only nuclear immunofluorescence, whereas the cells from which the other forms of the enzyme are isolated show some cytoplasmic immunofluorescence (preparations 6–10). The correlation of the patterns of immunofluorescence with enzyme molecular structure is certainly intriguing, but will require considerably more examples before we can construct a statistical norm for each type of cell. The similar kinetic properties of terminal transferases from thymus and leukemic blasts suggested that they could be derived from the same precursor molecule. It is also possible that terminal transferase occurs in multiple isozymic forms unrelated or distantly related in amino acid sequence. Direct evidence supporting close similarity in structure was obtained immunochemically. Precipitating and neutralizing antibodies to both calf and human terminal transferase were raised in rabbits. Generally, 5–20 μg of protein of either rabbit anti-calf terminal transferase or rabbit anti-human terminal transferase will give 50% inhibition of 1 U of the homologous terminal transferase. In the presence of excess antibody, inhibition of human and calf enzyme activity is >95%, when rabbit antibody to either antigen is used. Data for inhibition of each form of terminal transferase by rabbit anti-calf, rabbit anti-human, and goat anti-human terminal transferase are shown in Table IV. The titers of antisera raised in goats were significantly lower than those obtained in rabbits, despite repeated booster immunizations. Only one of two immunized goats produced antiserum. Control sera from six unimmunized rabbits and two unimmunized goats substantially inhibited terminal transferase activity when mixed with tissue extracts containing the enzyme. This inhibition was eliminated by passing sera over Chelex-100 resin and then diluting it 1:3 with 5 mg/ml bovine serum albumin in phosphate-buffered saline. Although the inhibitor has not been identified, it is probably one or more metal ions. It is interesting to note that although rabbit antisera to the calf enzyme neutralize human terminal transferase slightly better than calf terminal transferase, rabbit antisera to the human enzyme neutralize human terminal transferase from four to seven times more effectively than calf terminal transferase. Goat antiserum to the human enzyme neutralizes all molecular forms of the human enzyme, whereas it has virtually no effect on the enzyme from calf thymus. Each type of human terminal transferase, regardless of polypeptide structure, is neutralized nearly identically by each type of antiserum. Thus, the human enzymes with M_r = 62,000, 42,500, and 27,000/10,000 show identical patterns of neutralization as shown in Fig. 3. 1 U of each type of terminal transferase activity is inhibited by 5–7 μg of antiserum protein directed against the calf thymus enzyme in rabbits, by 10–21 μg antiserum protein directed against the human enzyme in rabbits and by 56–85 μg of antiserum protein directed against the human enzyme in goats.

Ouchterlony microdiffusion analyses of two types of antisera and four types of antigens are shown in Fig. 4. Precipitating antibody is contained in each serum. Single precipitin lines without spurs are observed in reactions of antigenic identity when all forms of human terminal transferase are diffused against both types of antisera. Spurs are observed when calf and human terminal transferase are compared so terminal transferases from the two species are immunochemically different (Fig. 4).

Immunofluorescence studies were carried out using the sera directly, as well as specific antibody preparations that had been purified by chromatography on antigen affinity columns. Cells were obtained from human thymus. Most terminal transferase antigen was in the cytoplasm, as was also observed in rat thymocytes (9). Satisfactory fluorescence was obtained with 0.60 μg of IgG per slide of affinity column purified rabbit anti-

TABLE IV
Inhibition of Terminal Deoxynucleotidyl Transferase Activity by Antisera

Serum	Serum protein required for 50% neutralization of 1 U of terminal transferase activity			
	Human lymphoblast (M_r = 62,000)	Human lymphoblast (M_r = 42,500)	Human thymus (M_r = 27,000 & 10,000)	Calf thymus (M_r = 30,500 & 9,500)
	μg	μg	μg	μg
Rabbit anti-human terminal transferase	20.8	14.2	10.2	75.4
Rabbit anti-calf terminal transferase	6.7	6.7	5.6	11.3
Goat anti-human terminal transferase	83.3	84.2	56.0	>500

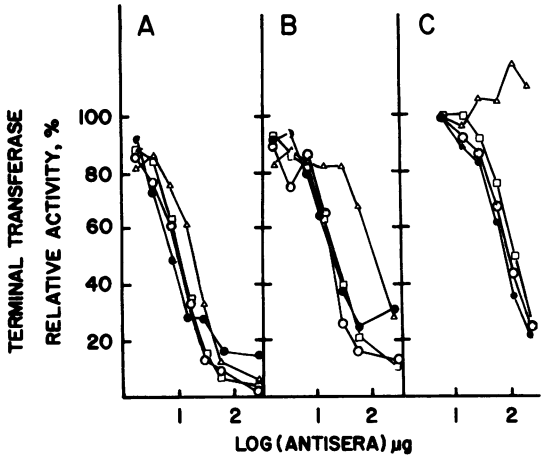

FIGURE 3 Neutralization of terminal transferase by antisera: (*A*) rabbit anti-calf thymus terminal transferase (*B*) rabbit anti-human terminal transferase (*C*) goat anti-human terminal transferase. Enzyme (~1 U) was incubated for 15 h at 4°C in the presence of each antiserum (serially diluted with 5 mg/ml bovine serum albumin in phosphate-buffered saline). The antigen-antibody mixture was tested directly for terminal transferase activity according to the assay protocol described in Methods. (○) 1.66 U of terminal transferase from human thymus (M_r = 27,000/10,000) (preparation 10). (△) 1.59 U of terminal transferase from calf thymus (M_r = 30,500/9,500) (preparation 11). (●) 0.96 U of terminal transferase from the lymphoblasts of a patient with ALL (M_r = 62,000) (preparation 2). (□) 1.40 U of terminal transferase from the blast-infiltrated spleen of a patient with ALL (M_r = 42,500) (preparation 4).

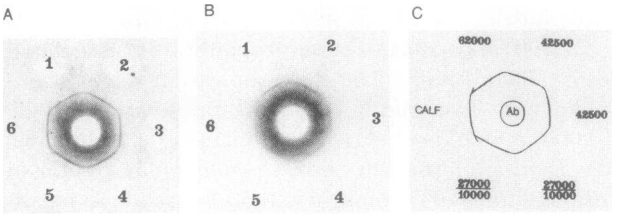

FIGURE 4 Microdiffusion of rabbit antisera against purified calf and human terminal transferase. (*A*) Rabbit anti-calf terminal transferase serum diluted 1:1 with phosphate-buffered saline (center well) (*B*) Rabbit anti-human terminal transferase serum diluted 1:1 with phosphate-buffered saline (center well). The antigens tested in A and B (50–500 ng) were: Well No. 1, human terminal transferase (M_r = 62,000), preparation 2 (Table III). Well No. 2, human terminal transferase (M_r = 42,500), preparation 4 (Table III). Well No. 3, human terminal transferase (M_r = 42,500), preparation 6 (Table III). Well No. 4, human terminal transferase (M_r = 27,000/10,000), preparation 8 (Table III). Well No. 5, human terminal transferase (M_r = 27,000/10,000), preparation 9 (Table III). Well No. 6, calf thymus terminal transferase (M_r = 30,500/9,500), preparation 11 (Table III). All wells contained 20 μl sample and diffusion occurred for 15 h at room temperature. Bands were stained with 0.1% Coomassie Brilliant Blue R-250. (*C*) An artist's perception of the precipitin lines on the microdiffusion plates, A and B.

serum to human terminal transferase as compared with 5.2 μg IgG per slide of affinity column purified rabbit antiserum to calf thymus terminal transferase. Our antiserum to the calf thymus enzyme is similar in potency to sera employed by others (4).

DISCUSSION

The purpose of this study was to purify and determine the structure of human terminal transferase in the two major types of leukemia in which the enzyme is commonly found. Terminal transferase is present in high levels in the majority of non-T, non-B ALL and T-cell ALL and in 40% of patients with CML in the accelerated phase of disease (4). From adult non-T, non-B ALL, we recently purified terminal transferase and determined that the protein existed in SDS as a single polypeptide of M_r = 62,000 (11). This structure was in contrast to all the previous findings with the only other common source of terminal transferase, calf thymus (12). The calf thymus enzyme in SDS exists as a smaller molecule with two nonidentical subunits (12). Since the kinetic properties of each form of transferase appear to be nearly identical (21, 26), the hypothesis (9, 11, 21, 28) that a high molecular weight form of terminal transferase might be cleaved to a smaller form as hematopoietic cells differentiate, obtains additional credibility. Therefore, we have purified terminal transferase from a number of different types of leukemic cells, and from human and calf thymus tissue.

By isolating the purified enzyme from human thymus, we demonstrate in SDS gels that the molecular weight differences we have previously seen between terminal transferases from human lymphoblasts and calf thymus may be tissue related. The subunit structures of the enzymes from thymus of calf and human origin are comparable. Each has two nonidentical subunits of molecular weight 27,000 and 10,000 (human) and 30,500 and 9,500 (calf). Terminal transferase from leukemic blasts of two patients with null cell ALL differed from the thymic enzyme and is a single polypeptide of M_r = 62,000. In the cells of two patients with untyped ALL, a subunit polypeptide of 42,500 was obtained. From the cells of patients with CML in blast crisis, two types of subunit structure were observed: (*a*) a M_r = 42,500 polypeptide subunit and (*b*) two nonidentical subunits of M_r = 27,000 and 10,000. Since not all of the CML patients had typing of cells, we are unable to ascribe a molecular weight structure to a particular type of CML blast. However, most patients with CML in blast crisis are typically null in type rather than T marked (29). Therefore, one possible discrepancy in our hypothetical assignment of terminal transferase molecular structure to cell type is that we might have predicted that the enzyme isolated from CML blasts would be of the largest molecular weight, 62,000. Alternatively, it is possible that the state of dif-

ferentiation of null cell ALL and blastic CML are not as similar as marker data suggest.

More direct assignment of terminal transferase to cell type is hindered by our inability to isolate enough early normal progenitor cells of defined type (4, 30) for purification of terminal transferase in sufficient quantities for structural investigations. However, there is evidence of phenotypic similarity between terminal transferase containing cells from normal marrow and lymphoblasts in common acute lymphoblastic leukemia (30). Combined immunological assays for terminal transferase and membrane markers have shown that terminal transferase containing-cells in nonleukemic human bone marrow carry ALL-associated and Ia-like antigens, but no thymocyte markers or surface immunoglobulins. Normal terminal transferase containing cells in marrow could be the precursors involved in common non-B, non-T acute lymphoblastic leukemia. Under these circumstances the structure of terminal transferase may very well be identical in normal bone marrow lymphoid precursurs and blasts from null cell ALL.

Terminal transferases from thymus and lymphoblasts are catalytically and immunochemically similar. All of the molecular species are neutralized by the same antisera and give reactions of antigenic identity to one another when tested by immunodiffusion on Ouchterlony plates. They must be very similar in amino acid sequence. Although the biological function of terminal transferase is not known, it may act as a "somatic mutagen" to generate immunological diversity (5, 6). Since this postulated mechanism of action requires activity during the processing of DNA, the active form of the enzyme is likely to be associated with the intranuclear species. By immunofluorescence studies, terminal transferase is located exclusively in the nucleus in marrow cells and in cells from ALL (10). We have detected terminal transferase in blastic CML cells in both the nucleus and cytoplasm by immunofluorescence (27). Goldschneider et al. (8) have reported a cytoplasmic location for terminal transferase protein in mouse thymocytes, and Janossy et al. (31) have demonstrated a predominantly nuclear location in human thymocytes. Isolation of the purified proteins that we report here demonstrate that regardless of the source or molecular weight, the protein is catalytically active and appears to be homogeneous in structure. Therefore, we cannot rule out the possibility that the cytoplasmic form of the enzyme plays an, as yet, undefined role in cellular metabolism.

The in vitro isolation of the different molecular forms of human terminal transferase we have described is not necessarily evidence for processing pathways in vivo. Increased levels of proteolytic enzymes in CML blasts and thymocytes relative to levels in ALL blasts offer an explanation for our observations. We have attempted to eliminate proteolysis as a problem during the purification of the enzyme from cells of one patient with CML in blast crisis, and from human thymus, by the addition of PMSF, and by the development of rapid purification schemes. In our experiments using PMSF and CML blast cells, we have found no evidence for the presence of a M_r = 62,000 form of the enzyme. We must conclude that a systematic investigation of the origin of multiple forms of terminal transferase should include an examination of physiological processing, artifactual degradation, or multiple isozymes coded by different genes for terminal transferase.

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