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Research Article

In the present study, terminal deoxynucleotidyltransferase was examined in the peripheral blood and (or) bone marrow of 115 children with a variety of neoplastic, hematologic, and other unrelated disorders. Terminal deoxynucleotidyltransferase activity was present at 4.08+/-0.74 U/108 cells in 23 morphologicall normal bone marrow samples from childhood controls. Terminal transferase was present at greater than 23 U/108 nucleated cells and at greater than31 U/108 blasts in the bone marrow of all children with acute lymphoblastic leukemia studied at initial diagnosis and at disease relapse. Terminal deoxynucleotidyltransferase was detectable at low levels, less than 7.5 U/108 cells, in all remission marrow smaples. Bone marrow terminal transferase activity was markedly elevated in all untreated acute lymphoblastic leukemia patients, whereas low levels which were difficult to interpret were present in the peripheral blood samples of two patients at diagnosis and six patients at relapse who had low absolute lymphoblast counts. Because of greater variation in the lymphoblast content of peripheral blood, bone marrow assays are more reliable in detecting disease activity. Marrow terminal deoxynucleotidyltransferase values obtained during the active phase of acute lymphoblastic leukemia were significantly greater than those found in other types of leukemia, bone marrow malignancies, and hematologic disorders. Terminal transferase determinations in blast cells of two patients with leukemic conversion of non-Hodgkin's lymphoma and in tumor cells from one patient […]

Terminal Deoxynucleotidyltransferase Distribution in Neoplastic and Hematopoietic Cells

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ABSTRACT In the present study, terminal deoxynucleotidyltransferase was examined in the peripheral blood and (or) bone marrow of 115 children with a variety of neoplastic, hematologic, and other unrelated disorders. Terminal deoxynucleotidyltransferase activity was present at 4.08±0.74 U/10^8 cells in 23 morphologically normal bone marrow samples from childhood controls. Terminal transferase was present at >23 U/10^8 nucleated cells and at >31 U/10^8 blasts in the bone marrow of all children with acute lymphoblastic leukemia studied at initial diagnosis and at disease relapse. Terminal deoxynucleotidyltransferase was detectable at low levels, <7.5 U/10^8 cells, in all remission marrow samples. Bone marrow terminal transferase activity was markedly elevated in all untreated acute lymphoblastic leukemia patients, whereas low levels which were difficult to interpret were present in the peripheral blood samples of two patients at diagnosis and six patients at relapse who had low absolute lymphoblast counts. Because of greater variation in the lymphoblast content of peripheral blood, bone marrow assays are more reliable in detecting disease activity. Marrow terminal deoxynucleotidyltransferase values obtained during the active phase of acute lymphoblastic leukemia were significantly greater than those found in other types of leukemia, bone marrow malignancies, and hematologic disorders. Terminal transferase determinations in blast cells of two patients with leukemic conversion of non-Hodgkin's lymphoma and in tumor cells from one patient with Burkitt's lymphoma were within the control range. These data further define the usefulness of terminal deoxynucleotidyltransferase assay in the differentiation and classification of hematologic malignancies.

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INTRODUCTION

Recent evidence suggests that terminal deoxynucleotidyltransferase (TdT)[1] may be a biochemical marker useful for classifying leukemias (1–6). This unusual deoxynucleotide-polymerizing activity is normally present at high levels only in the thymus (7) and at low levels in the bone marrow (5), but has not been detected in circulating or mitogen-stimulated lymphocytes (5). Identification of TdT activity in circulating blast cells (1–6) and cell lines (8–10) from acute lymphoblastic leukemia (ALL) patients, and in some cases of chronic myelogenous leukemia in blastic crisis (2, 6, 10–12), has raised the speculation that it may be localized in a primitive hematopoietic stem cell (5). In vitro, terminal transferase catalyzes the addition of deoxynucleoside triphosphates to the 3′-hydroxyl ends of oligo- or polydeoxynucleotide initiators without template instruction (13). Since an activity of this kind might cause modification of DNA sequence, it has been suggested that the enzyme could participate in somatic diversification of lymphoid precursors during differentiation (13–15).

TdT levels in peripheral blood lymphoid cells of patients with ALL at different phases of the disease have been reported (4). The purpose of the present study was to examine TdT activity in bone marrow and peripheral blood from children with acute leukemia of various cell types, solid tumors, nonneoplastic hematologic disorders, and other unre-

[1] *Abbreviations used in this paper*: ALL, acute lymphoblastic leukemia; E rosettes, sheep erythrocyte rosettes; HBSS, Hanks' balanced salt solution; [^{3}H]dGTP, tritium-labeled deoxyguanosine triphosphate; Ig, immunoglobulins; ITP, idiopathic thrombocytopenia purpura; poly d(pA)$_{50}$, polymer of deoxyadenylic acid, average chain length 50 residues; TdT, terminal deoxynucleotidyltransferase.

lated conditions in which a bone marrow examination was included in the patient's diagnostic evaluation. The results to be reported here establish the range of TdT activity normally present in the bone marrow of children of varying ages, and define the specificity of the enzyme in differentiating neoplastic disease.

METHODS

Patients. Peripheral blood lymphoid cells and (or) bone marrow cells were examined in 62 children with ALL. 20 of the patients were studied initially at diagnosis; 18 patients were in complete disease remission on chemotherapy; 12 patients were in complete disease remission and off chemotherapy; and 12 patients were evaluated during disease relapse. Patients were treated according to a standardized ALL chemotherapy protocol (4) consisting of induction therapy, central nervous system prophylaxis, and multiple agent maintenance therapy continuing for 3 yr or until relapse.

Additional blood, bone marrow, and (or) tumor specimens were assayed from 8 children with other types of leukemia, 2 with Hodgkin's disease, 2 with non-Hodgkin's lymphoma and leukemic transformation, 1 with Burkitt's lymphoma, 2 with disseminated neuroblastoma, 2 with disseminated rhabdomyosarcoma, 5 with aplastic anemia, 8 with idiopathic thrombocytopenic purpura (ITP), and 23 with nonhematologic disorders.

Isolation of cells. 20–40 ml of peripheral blood and (or) 2.5–3 ml of iliac crest bone marrow was collected in 5 mM EDTA for assays of cell markers and terminal transferase activity. Peripheral blood and bone marrow samples were diluted 5:1 with 6% dextran (250,000 mol wt) in saline and were allowed to sediment 45 min at room temperature. Suspensions of nucleated cells from peripheral blood samples were centrifuged, diluted in Hank's balanced salt solution (HBSS), layered onto Ficoll-Hypaque (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) gradients, and then centrifuged at 250 *g* for 35 min according to the method of Boyum (16). The cells were aspirated from the interface and washed twice in HBSS. Contaminating monocytes were removed using carbonyl iron powder as described by Raff (17). An aliquot of the purified lymphoid cells was collected on glass slides using a cytocentrifuge and the cells were stained with Wright-Giemsa. Some cells appeared damaged by the gradient separation and preservation of morphological detail was such that mature and leukemic lymphoid cells could not always be distinguished although contamination of the lymphoid fraction by monocytes or polymorphonuclear leukocytes was easily monitored. Bone marrow cells were separated by dextran sedimentation as described for peripheral blood, but were not processed on Ficoll-Hypaque gradients. An aliquot of purified nucleated cells from marrow and (or) blood was counted and processed directly for assay of cell surface markers. Another aliquot was stored as a cell pellet at −70°C for later TdT assay.

Sheep erythrocyte (E) rosette assay. E-rosette assays were performed in duplicate according to the method of Bentwich et al. (18). 0.1 ml of lymphoid cells suspended in HBSS (5×10^6/ml) was incubated with an equal volume of a 0.5% suspension of fresh sheep erythrocytes in HBSS and 0.02 ml of human AB+ serum (previously absorbed with sheep erythrocytes) at 37°C for 5 min. The mixture was centrifuged at 50 *g* for 5 min and then incubated at 4°C for 1 h. After gentle resuspension, 200 cells were enumerated under oil immersion. Lymphoid cells with three or more adherent sheep erythrocytes were considered positive for E-rosette formation.

Determination of surface immunoglobulins. Surface immunoglobulins (Ig) were determined as previously described by Gajl-Peczalska et al. (19). In this procedure, 0.1 ml of lymphoid cells in HBSS (5×10^6/ml) were incubated with a 1:20 dilution of fluorescein-conjugated type-specific antisera at 4°C for 1 h. IgG, IgM, and IgA antisera were supplied by Dr. Neil Davis, Cincinnati Children's Research Foundation, Cincinnati, Ohio. After centrifugation, the cells were washed twice in cold phosphate-buffered saline (PBS) and the cell pellet was resuspended in 20 μl of 50% glycerol in PBS. 200 cells were enumerated using a Zeiss fluorescent microscope equipped with a vertical illuminator (Carl Zeiss, Inc., Yew York). The percentage of cells with surface Ig reported in the tables represents the sum of percentages of cells reacting with antisera to IgG, IgM, and IgA.

Enzyme assay. Lymphoid cells were suspended at a density of $1-2 \times 10^8$ cells/ml in 0.25 M potassium phosphate buffer, pH 7.5, containing 1 mM mercaptoethanol. The cell suspension was sonically disrupted by four 15-s bursts with cooling. The extract was centrifuged at 100,000 *g* for 60 min and the resulting supernatant fraction was then assayed directly for TdT.

Radioactive deoxynucleoside triphosphates were purchased from New England Nuclear, Boston, Mass. and were received in 50% ethanol solution. Unlabeled deoxynucleoside triphosphates were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. The radioactive deoxynucleoside triphosphate was diluted to the desired specific activity, taken to dryness to remove ethanol, and then dissolved in H_2O to a concentration of 5 mM before use. Polymer of deoxyadenylic acid (poly $d[pA]_{\overline{50}}$) was prepared in our laboratory (20). Assays were initially carried out with tritium-labeled deoxyguanosinetriphosphate ([^{3}H]dGTP) diluted to a spec act of 100 cpm/pmol. Samples with less than 1 U/10^8 cells are reassayed at a [^{3}H]dGTP spec act ≅1,000 cpm/pmol to confirm the presence of TdT activity.

Reaction mixtures for TdT contained 0.2 M potassium cacodylate buffer (pH 7.0), 1 mM [^{3}H]dGTP with a spec act of 100 or 1,000 cpm/pmol, 0.02 mM poly $d(pA)_{\overline{50}}$, 1 mM mercaptoethanol, and 15 μl of cell extract in a final volume of 125 μl. The reaction mixture was placed in a water bath at 35°C and started by addition of cell extract. 25-μl aliquots were removed at 6, 12, 18, and 24 min and placed on GF/C glass fiber disks (Whatman, Inc., Clifton, N. J.). Further processing of the reaction products (21) and calculation of activity have been described (22). 1 U of terminal transferase activity equals 1 nmol of dGTP polymerized in 1 h. Specific activities are expressed as units/10^8 nucleated cells. In Tables I and IV, TdT activities in peripheral blood and bone marrow are also calculated in terms of units/10^8 blasts. For peripheral blood, these calculations use estimates of the percentage of blasts in the lymphoid fraction where available, and assume that all TdT activity is in blasts and none is in mature lymphocytes.

Kinetic analysis of enzyme activity is essential for recognizing an occasional high background found in the crude extracts. Linear formation of product as a function of time is required for the judgment that a sample contains enzyme activity. Since antiserum to homogeneous calf thumus TdT gives essentially complete inhibition of activity in crude extracts (23), the assay is now assumed to be specific for this enzyme.

TABLE I
ALL at Initial Diagnosis

Patient	Age	Peripheral blood: Leukocyte count/mm³	Peripheral blood: Blasts	Peripheral blood: TDT U/10⁸ cells	Peripheral blood: TDT U/10⁸ blasts	Peripheral blood: Surface markers E	Peripheral blood: Surface markers Ig	Bone marrow: Blasts	Bone marrow: TdT U/10⁸ cells	Bone marrow: TdT U/10⁸ blasts	Bone marrow: Surface markers E	Bone marrow: Surface markers Ig
	yr		%			%		%			%	
1	6	25,400	78	299.3	374.1	10	10	99	374.0	377.8	2	6
2	3	2,200	5	4.5	90.0	47	28	90	262.1	291.2	14	4
3	3	21,500	45	186.0	395.7	18	8	95	232.8	245.1	7	7
4	8	53,200	70	133.0	184.7	10	1	99	160.3	161.9	5	0
5	3	1,430	26	ND	—	24	ND	78	150.0	192.3	ND	ND
6	8	5,000	14	18.8	104.4	21	ND	93	133.0	143.0	1	ND
7	8	132,500	72	136.1	181.5	6	1	95	131.0	137.9	2	1
8	2	15,200	58	44.6	73.1	10	21	99	111.0	112.1	5	21
9	5	89,000	63	81.0	95.3	81	19	80	ND	—	51	ND
10	7	299,000	93	53.1	56.5	13	3	76	72.7	95.7	32	2
11	9	8,400	68	68.0	98.6	12	4	97	ND	—	ND	ND
12	3	15,250	37	12.8	34.6	11	22	73	57.8	79.2	ND	ND
13	7	70,000	48	45.6	82.9	66	12	66	51.1	77.4	75	7
14	18	19,000	57	28.3	39.9	7	12	86	50.7	59.0	ND	ND
15	10	107,000	94	35.0	35.7	80	2	91	45.3	49.8	ND	ND
16	3	24,400	62	52.8	81.2	16	8	98	40.5	41.3	ND	ND
17	5	4,100	43	5.6	13.0	29	8	90	40.2	44.7	2	ND
18	2	89,500	65	44.3	59.1	11	7	22	32.4	147.2	12	8
19	2	18,300	79	64.5	79.6	23	2	95	30.4	32.0	ND	ND
20	4	14,000	39	ND	—	ND	ND	95	30.1	31.7	11	0
Terminal transferase activities:												
Range				4.5–299.3	13.0–395.7				30.1–374.0	31.7–377.8		
Mean±SE				73.0±17.5	115.6±25.0				111.4±22.6	128.9±22.8		
Controls, age 1 wk–14 yr. Number of specimens: peripheral blood, 11, bone marrow, 23.												
Range				<0.05–1.61					0.78–15.80			
Mean±SE				0.35±0.18					4.08±0.74			

ND, not done. Only Patients 13 and 15 had radiologic evidence of mediastinal mass.

RESULTS

ALL at diagnosis. The observations in 20 patients with ALL, before initiation of chemotherapy, are summarized in Table I. The mean age of the patients was 5.8 yr and the male to female ratio was 1:1. Bone marrow lymphoblasts varied from 22 to 99% (mean 86%) and peripheral blood lymphoblasts ranged from 5 to 94% (mean 56%) with the absolute lymphoblast count varying from 110 to 278,000/mm³. TdT activity was markedly elevated in bone marrow samples from all patients examined at diagnosis, (range 30.1–374.0 U/10⁸ cells, mean±SE = 111.4±22.6 U/10⁸ cells). When TdT activities in marrow were calculated in terms of blasts rather than nucleated cells, the range was 31.7–377.8 U/10⁸ blasts, mean ±SE = 128.9±22.8 U/10⁸ blasts. TdT activity was markedly elevated (12.8–299.3 U/10⁸ cells, mean ±SEM = 81.5±18.7 U/10⁸ cells) in lymphoid cells from 16 of the 18 peripheral blood samples examined. Lower levels of TdT activity were observed in patients 2 and 17 (Table I) who had blast counts of 110 and 1,763/mm³ and TdT values of 4.5 and 5.6 U/10⁸ cells, respectively. However, a TdT level of 18.8 U/10⁸ cells was detected in patient 6 in whom the peripheral lymphoblast count was only 700/mm³.

TABLE II
All Patients in Remission on Chemotherapy

Patient	Age at diagnosis	Remission duration	TdT	
			Peripheral blood	Bone marrow
	yr	*mo*	*U/10⁸ cells*	
1	12	36	1.40	2.30
2	5	34	0.19	ND
3	10	34	0.62	0.99
4	4	29	0.33	0.83
5	3	24	0.48	1.37
6	5	23	0.81	2.70
7	3	21	0.57	7.50
8	3	21	0.59	3.09
9	3	17	1.53	2.15
10	3	17	1.04	2.20
11	5	15	0.45	1.40
12	8	13	<0.05	2.40
13	4	12	1.60	4.20
14	4	12	0.40	0.96
15	3	12	<0.05	7.10
16	7	10	0.68	2.15
17	4	9	ND	3.70
18	2	2	0.50	2.13
Terminal transferase activities:				
Range			<0.05–1.60	0.83–7.50
Mean±SE			0.66±0.11	2.77±0.47

ND, not done. No blasts were seen on peripheral blood smears and the percentage of blasts in the marrow was <5, so TdT values are reported as units/10^8 nucleated cells and no correction for percentage of blasts could be made.

These values indicate that TdT activity varies widely in lymphoblast populations. When TdT activities in peripheral blood were calculated in terms of blasts rather than nucleated lymphoid cells, the range was 13.0–395.7 U/10^8 blasts, mean±SE = 115.6±25.0 U/10^8 blasts. There was a significant correlation ($r = 0.73, P < 0.01$) between TdT activity in peripheral lymphoid cells and activity in bone marrow.

The mean levels of TdT activity observed in peripheral blood and bone marrow samples of all ALL patients at diagnosis were significantly greater ($P < 0.005$ and $P < 0.001$, respectively, Student's t test) than the mean peripheral blood activity found in 11 childhood controls (range <0.05–1.61 U/10^8 cells, mean±SE = 0.35±0.18 U/10^8 cells) and the mean activity observed in "normal" bone marrow samples from 23 children without evidence of malignancy or primary hematologic disorders (range 0.78–15.80 U/10^8 cells, mean±SE = 4.08±0.74 U/10^8 cells).

Cell surface marker determinations were performed on blood and (or) bone marrow lymphoid cells obtained from ALL patients before initiating chemotherapy. We have designated 3 of the 20 patients to be T-cell leukemia (patients 9, 13, and 15, Table I) since 81, 66, and 80% of the peripheral blood cells (containing 63, 48, and 94% lymphoblasts) formed E rosettes. Bone marrow samples were examined in patients 9 and 13, and 51 and 75% E rosettes were present in samples containing 80 and 66% lymphoblasts, respectively. These three cases represent significant (>50%) rosette formation by lymphoblasts. All three patients had initial lymphoblast counts greater than 25,000/mm³ and two of three patients had a mediastinal mass. In relatively pure populations of blood or bone marrow lymphoblasts, the morphologic identification of normal vs. neoplastic lymphoid cells possessing surface markers offers little difficulty. However, in mixed populations both lymphocytes and lymphoblasts may react in the test system, and assignment of surface markers to lymphoblasts is difficult. Wright-Giemsa staining of cytocentrifuge preparations of the rosetting cells does not entirely alleviate this problem. The absolute distinction between a rosetting normal lymphocyte and a rosetting microlymphoblast is

extremely difficult in some patients. We have designated 17 of the 20 patients as "null cell" leukemia although a small percentage of the total bone marrow and blood lymphoid populations did contain E-rosetting or surface immunoglobulin-bearing cells. This small percentage of surface marker positive cells can be accounted for by the reaction of normal lymphocytes in the test system. Of the 17 patients designated as null ALL, none had a mediastinal mass and only 4 of 17 had greater than 25,000 peripheral lymphoblasts/mm^3 at diagnosis. These findings are consistent with previous reports which illustrate the heterogeneity of cell surface markers in ALL and indicate that "bulk disease" is more frequently observed in patients with T-cell leukemia (24–26).

No significant differences were observed between mean peripheral blood or bone marrow TdT values of null and T-cell leukemias, between mean peripheral blood or bone marrow TdT values of patients less than 10 yr of age or 10 yr and older, or between mean peripheral blood or bone marrow TdT values in patients with low and high lymphoblasts counts, i.e., <25,000 vs. >25,000/mm^3.

ALL after induction chemotherapy. After 4–6 wk of induction chemotherapy and at the time hematologic remission was achieved, TdT assays were again performed on samples of peripheral blood in 13 patients and on bone marrow in 11 patients. The enzyme activity had declined markedly in all samples assayed and TdT values ranged from <0.05–3.38, mean ±SE = 1.16±0.26 U/10^8 cells in bone marrow and from <0.05–2.08, mean±SE = 0.85±0.16 U/10^8 cells in peripheral blood (individual data not presented). There was no correlation between initial TdT levels and TdT levels obtained after 4–6 wk of induction chemotherapy. Although no significant difference was observed between mean peripheral blood TdT levels of patients after induction therapy and controls, the mean bone marrow activity after induction therapy was significantly lower than that observed in the control group (1.16 vs. 4.08, $P < 0.02$). This lower TdT activity could be caused by decrease in the steroid-sensitive TdT-containing cells as has been demonstrated experimentally (27, 28).

ALL during disease remission. TdT activity was determined in samples of peripheral blood and (or) bone marrow in 18 children during disease remission while continuing on maintenance chemotherapy (Table II). These patients had been in continuous remission for 2–36 mo at the time assays were performed. The average TdT levels in peripheral blood and bone marrow cells did not differ significantly from values obtained in control peripheral blood cells and bone marrow cells. Bone marrow levels ranged from 0.83–7.50 U/10^8 cells, mean±SE = 2.77±0.47 U/10^8 cells and peripheral blood levels ranged between <0.05 and 1.60 U/10^8 cells, mean±SE = 0.66 ±0.11 U/10^8 cells.

TABLE III
All Patients in Remission, off Chemotherapy, after 3 Yr

Patient	Age at diagnosis	Remission duration	TdT	
			Peripheral blood	Bone marrow
	yr	*mo*	*U/10^8 cells*	
1	3	161	0.43	ND
2	8	134	0.72	0.78
3	5	101	0.47	ND
4	9	97	0.30	ND
5	8	81	0.58	1.43
6	6	66	0.27	4.42
7	5	60	1.20	ND
8	4	53	0.76	ND
9	2	47	0.49	1.04
10	10	45	1.30	5.06
11	6	44	0.36	0.97
12	9	37	0.84	ND
Terminal transferase activities:				
Range			0.27–1.30	0.78–5.06
Mean±SE			0.64±0.10	2.28±0.79

ND, not done. See footnote to Table II. No blasts were observed in the peripheral blood and the percentage of blasts in bone marrow was <5.

TABLE IV
ALL at Disease Relapse

Patient	Age at diagnosis	Months after diagnosis	Peripheral blood: Leukocyte count mm³	Peripheral blood: Blasts	Peripheral blood: TdT U/10⁸ cells	Peripheral blood: TdT U/10⁸ blasts	Bone marrow: Blasts	Bone marrow: TdT U/10⁸ cells	Bone marrow: TdT U/10⁸ blasts
	yr			%			%		
1	4	64	14,000	72	67.8	91.6	98	108.0	110.2
2	2	57	3,800	0	1.9	—	84	145.0	172.6
3	3	46	2,700	0	4.8	—	81	161.7	199.6
4	4	44	5,300	10	60.8	202.7	61	270.0	442.6
5	11	26	2,500	1	5.5	110.0	97	251.0	258.8
6	4	23	4,300	4	10.1	101.0	72	54.1	75.1
7	4	20	5,900	24	21.0	31.3	92	44.0	47.8
8	5	19	2,500	3	3.5	38.9	89	1,000.0	1,123.6
9	7	11	45,100	30	12.3	38.4	74	ND	—
10	17	10	6,000	28	147.0	358.5	86	222.0	258.1
11	8	10	22,900	22	58.2	215.6	77	67.2	87.3
12	5	8	14,500	2	2.2	27.5	67	23.8	35.5
Terminal transferase activities:									
Range					1.9–147.0	27.5–358.5		23.8–1000.0	35.5–1123.6
Mean±SE					32.9±12.6	121.6±34.0		213.3±82.8	255.6±94.0

ND, not done. Values of TdT activity are reported both as units/10⁸ nucleated cells and units/10⁸ blasts. Where the percentage of blasts is very low, the calculated activity of TdT per 10⁸ blasts may not be reliable because of the very large correction factor used to convert from units/10⁸ nucleated cells to units/10⁸ blasts.

The observations in 12 additional children with ALL who were in disease remission after 3 yr of continuous chemotherapy are summarized in Table III. At the time of study, all of these children were off chemotherapy and had been in remission for 37–161 mo. TdT activity in peripheral lymphoid cells of 12 patients ranged from 0.27–1.30 U/10⁸ cells, mean±SE = 0.64±0.10 U/10⁸ cells and in bone marrow samples from 6 patients, TdT values ranged from 0.78–5.06 U/10⁸ cells, mean±SE = 2.28±0.79 U/10⁸ cells. These values from peripheral blood and bone marrow cells did not differ significantly from those obtained from the group of patients in remission for 2–36 mo while on chemotherapy or from the controls.

ALL at disease relapse. 12 children with ALL were studied at the time of disease relapse and the findings are summarized in Table IV. TdT activity was markedly elevated in all bone marrow samples examined, 23.8–1,000.0 U/10⁸ cells (mean±SE = 213.3±82.8 U/10⁸ cells). TdT values in peripheral lymphoid cells ranged from 1.9–147.0 U/10⁸ cells (mean±SE = 32.9±12.6 U/10⁸ cells). Peripheral blood samples from five patients containing only 0–3% lymphoblasts had lower levels of TdT activity, 1.9–5.5 U/10⁸ cells, whereas corresponding bone marrow samples containing 67–97% blasts had TdT levels ranging from 23.80–1,000.0 U/10⁸ cells. When TdT activities were calculated in terms of blasts, the range for peripheral blood was 27.5–358.5 U/10⁸ blasts, mean±SE = 121.6± 34.0 U/10⁸ blasts; the range for bone marrow was 35.5–1123.6 U/10⁸ cells, mean±SE = 255.6±94.0 U/10⁸ cells. The mean peripheral blood and bone marrow TdT values at relapse did not differ significantly from the mean values of a different group of patients (Table I) at initial diagnosis, but were significantly higher than the mean peripheral blood and bone marrow values of controls ($P < 0.025$, $P < 0.001$).

Testicular relapse preceded hematologic relapse by 2 mo in patient 7 (Table IV) and TdT activity was 24.8 U/10⁸ cells isolated from the leukemic infiltrated testicular tissue at a time when bone marrow and blood TdT activities were within the control range. At the time of hematologic relapse, TdT levels of 21.0 and 44.0 U/10⁸ cells were found in peripheral blood and bone marrow, respectively.

Other leukemias of childhood. Results of TdT determinations in eight children with types of acute leukemia other than lymphoblastic are summarized in Table V. In these cases, the diagnosis of leukemia was based on routine morphologic criteria employing Wright's, Periodic-acid-Schiff, and Sudan black staining procedures and was made by different observers before, and independently of, enzyme deter-

minations. TdT activity was either undetectable or minimally elevated in all bone marrow and peripheral blood samples assayed. Bone marrow samples containing 43–74% blasts were examined in four patients with acute myeloblastic leukemia. The TdT activity ranged from 0.14 to 7.08 U/10^8 cells and the mean value of 2.97 did not differ significantly from the mean TdT value of control marrows, 4.08 U/10^8 cells. Of three patients with acute myeloblastic leukemia having 42–62% peripheral blood blasts, TdT activity ranged from 1.39–5.81 U/10^8 cells in peripheral blood samples and the mean of 2.91 U/10^8 cells differed significantly from the mean TdT level of controls, 0.35 U/10^8 cells ($P < 0.01$).

Three children with acute myelomonoblastic or monoblastic leukemia were examined when the peripheral blood blasts ranged from 54 to 93% and bone marrow samples contained 76–95% blasts. TdT activity was undetectable or low in extracts of both peripheral blood and bone marrow blasts, <0.05–3.46 and 1.62–3.52 U/10^8 cells, respectively. No significant differences were observed between the mean TdT values of peripheral blood or bone marrow samples from children with acute myelomonoblastic or monoblastic leukemia and controls.

One patient included in the classification "acute myelomonocytic leukemia" (Table V) had an acute leukemia undifferentiated in type by usual morphological and histochemical criteria. There were 38% peripheral blasts and 80% bone marrow blasts with respective TdT levels of 4.73 and 8.17 U/10^8 cells. Although these levels did not approach the mean levels observed in ALL patients, they were elevated above mean control values.

Malignancies other than leukemia. The results of TdT determination in two patients with non-Hodgkin's lymphoma with leukemic transformation occurring at 3 and 20 mo after initial presentation are summarized in Table V. Peripheral blood samples contained 7 and 97% blasts and bone marrow samples contained 76 and 92% blasts. Neither peripheral blood nor bone marrow samples in these patients contained TdT activity above the control range. TdT was detectable at 1.24 U/10^8 tumor cells derived from a biopsy of an abdominal Burkitt's lymphoma. TdT activity in two patients with Hodgkin's disease, stages IIB and IVB, two patients with stages III and IV neuroblastoma, and two patients with stage IV rhabdomyosarcoma was low (Table V). Bone marrow specimens from two patients, one with disseminated neuroblastoma and one with disseminated rhabdomyosarcoma (Table V), contained malignant cells and values of 0.81 and 4.76 U/10^8 cells were obtained, respectively. TdT activity was undetectable in cell preparations of neuroblastoma tissue obtained at the time of surgical exploration in one patient. The consistently low levels of TdT activity in neoplastic cells other than ALL blasts indicate the value of this assay in distinguishing malignant cell populations.

TABLE V
Hematologic Diseases Other Than ALL

Diagnosis*	No. patients	Age range	TdT	
			Peripheral blood	Bone marrow
		yr	*U/10^8 cells*‡	
Acute myelomonocytic leukemia	8	1.6–17	2.43±0.85	3.60±1.12
Lymphoma	4	3–14	0.58±0.18	2.16±0.75
Neuroblastoma	2	2–10	2.90, 1.50	0.81, 0.68
Rhabdomyosarcoma	2	4–13	0.44, 1.30	4.76, ND
ITP	8	2–12	0.35±0.07	4.85±1.15
Aplastic anemia	5	1–16	ND§	2.36±0.46

* Acute myelomonocytic leukemia includes acute myelogenous, myelomonocytic, and monocytic leukemias. Lymphoma includes two patients with stage IV non-Hodgkin's lymphoma with leukemic transformation and two patients with stages IIB and IVB Hodgkin's disease.

‡ Activities of TdT are expressed as mean±SE. Where only two patients were studied, individual values are presented.

§ Peripheral blood activity not determined because of low leukocyte count.

Other hematologic disorders. To determine the distribution of TdT activity in nonmalignant disorders, peripheral blood and marrow specimens were obtained for assay during the routine evaluation of other primary hematological conditions in children. Peripheral blood enzyme activity was measured in six of eight patients with ITP and was undetectable or low in all six children studied, <0.05–0.56 U/10^8 cells, mean±SE = 0.35±0.07 U/10^8 cells. TdT activity in bone marrow cells in the eight patients ranged from 1.20–12.00 U/10^8 cells, mean±SE = 4.85±1.15 U/10^8 cells (Table V). The highest bone marrow activity observed was 12.00 U/10^8 cells in a 2-yr-old male who has no evidence of hematological abnormalities 1 yr after spontaneous remission of ITP. Assays of bone marrow specimens from five children with aplastic anemia revealed a range of TdT activity from 1.21 to 3.40 U/10^8 cells, mean±SE = 2.36 ±0.46 U/10^8 cells (Table V). There was no significant correlation with age and values obtained in patients with ITP, and aplastic anemia did not differ significantly from the control group of hematologically normal patients.

Controls. Children 1 wk–14 yr of age requiring a bone marrow examination in their diagnostic evaluation and in whom no evidence of neoplastic or primary hematologic disorder was present were used as controls for this study. 23 bone marrow samples were examined and TdT activity was detected in all specimens, range 0.78–15.80 U/10^8 cells, mean±SE = 4.08

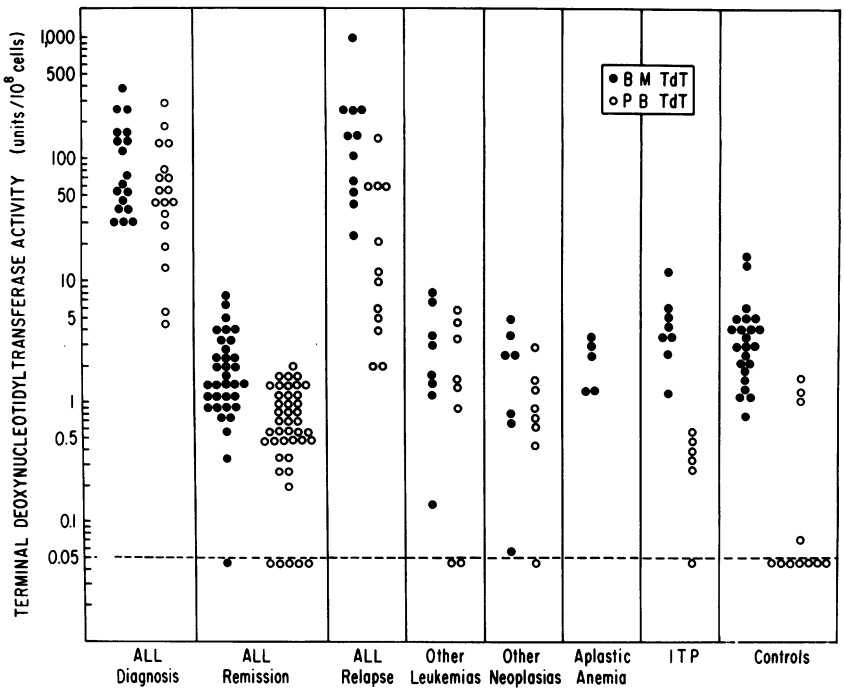

FIGURE 1 Summary of TdT activities in bone marrow (BM) and (or) peripheral lymphoid (PB) cells of children with ALL at different stages of the disease process, other neoplastic, hematologic, and unrelated disorders. The line at 0.05 U/10^8 cells shows the minimal detectable level of TdT activity. 1 U = 1 nmol of nucleotide incorporated/h per 10^8 cells at 35°C.

±0.74 U/10^8 cells (Table I). In 21 of the 23 marrow specimens assayed, transferase activity was <6.0 U/10^8 cells, whereas the other 2 samples contained activities of 13.29 and 15.80 U/10^8 cells. In both instances, bone marrow cellular morphology and content were normal by microscopic examination. The TdT level of 13.29 U/10^8 cells was present in a 2-yr-old female with a history of viral illness of undetermined etiology 3 wk before examination. The second patient was a 5-yr-old male with a progressive neurological disorder characterized by ataxia and developmental retardation. The TdT value in this child's bone marrow was 15.80 U/10^8 cells. The significance of higher values observed in these two children is uncertain and deserves continued follow-up and investigation in a larger series of normal marrows as well as marrows obtained for evaluation of hematologic and infectious diseases. There were no significant differences found between mean bone marrow TdT levels in groups of children ages 0–2, 2–10, and 10–12 yr. Extracts of peripheral blood lymphoid cells were assayed from 11 children and TdT activity was undetectable in 8 (<0.05 U/10^8 cells), and ranged from 0.07 to 1.61 U/10^8 cells in the remaining 3 (mean±SE = 0.35 ±0.18 U/10^8 cells).

A summary of TdT activity in bone marrow and peripheral lymphoid cells of children with ALL, other neoplastic, hematologic, and unrelated disorders is seen in Fig. 1.

DISCUSSION

Most clinical reports on TdT have dealt primarily with the distribution of enzyme activity in peripheral blood lymphoid cells (1–4). The purpose of this present study was to compare levels of TdT activity in bone marrow and peripheral lymphoid cells derived from children with various neoplastic, hematologic, and other unrelated disorders. The results confirm the presence of TdT in the normal bone marrow cell populations of children. Previous observations of strikingly increased levels of enzyme activity in ALL during active phase of the disease are extended to bone marrow and peripheral blood of a larger number of patients. The presence of low levels of bone marrow enzyme activity in other types of childhood neoplastic and hematologic disorders is demonstrated.

The cumulative data from the present series of 62 ALL patients and a previous group of 27 children with ALL (4) reveal significantly elevated TdT levels in bone marrow and (or) peripheral blood samples of all 42 patients examined at diagnosis or relapse. At the time of disease remission, TdT values in 47 children with ALL fall within the range observed for childhood controls. McCaffrey et al. (1, 2) also

reported TdT elevation in the peripheral blood of 12 of 14 patients (children and adults) with ALL. The nature of the leukemic process dictates that bone marrow enzyme assay should be more reliable than peripheral blood assay in detecting disease activity, since the percentage of blasts in marrow will always be high, whereas this may not occur in peripheral blood. The results obtained in this report indicate that bone marrow TdT levels are of greater value than assays of peripheral blood when the percentage of blasts in peripheral blood is low. A low peripheral blast count poses serious problems because of the large volumes of blood which are required to obtain sufficient numbers of blasts for assay.

Follow-up studies on a previously reported series of ALL patients (4) now reveal that four of the seven patients evaluated at diagnosis relapsed after 6–11 mo. Three of these patients were males with T-marker lymphoblasts, mediastinal masses, and initial blast counts greater than 50,000/mm^3. The fourth patient was a 17-yr-old female with null lymphoblasts and an initial blast count of 366,000/mm^3. In this group of four patients who relapsed, TdT levels ranged from 8 to 210 U/10^8 peripheral lymphoid cells at the time of diagnosis, indicating that TdT levels alone are not predictive of ultimate prognosis in ALL. Determinations of lymphoblast surface markers were performed at the time of relapse in two of the four patients from the previously reported series. Lymphoblast characteristics at relapse were unchanged from diagnosis in patient 10 (Table IV) with null lymphoblasts and in patient 11 (Table IV) with T-marker lymphoblasts predominating. Patient 7 (Table IV) who was not included in the initial series had T-marker lymphoblasts both at diagnosis and relapse in peripheral blood and testicular infiltrates. Combining data from both series of ALL patients, no statistical difference was found between the mean peripheral blood TdT values in T-lymphoblastic leukemia (40.9±8.8 U/10^8 cells) and null cell ALL (86.1±18.8 U/10^8 cells), $P > 0.10$.

Bone marrow TdT activity was markedly elevated at the time of diagnosis in all patients classified as ALL on the basis of cytological and cytochemical analysis. Morphologic classification of the cell type was assigned before obtaining the results of bone marrow TdT assay in every patient in this report. Bone marrow TdT activity in ALL at diagnosis was higher than bone marrow activity from children classified as acute myeloblastic leukemia, acute myelomonoblastic leukemia, acute undifferentiated leukemia, and in disseminated neuroblastoma and disseminated rhabdomyosarcoma. These biochemical findings indicate that TdT activity may be a useful adjunct in differentiating neoplastic bone marrow cell populations in children.

The observation of very low TdT activity in bone marrow blasts of two children with leukemic transformation of previously isolated non-Hodgkin's lymphoma and in tumor cells from a biopsy of Burkitt's lymphoma is evidence that TdT may also be a useful marker in differentiating malignant cell populations of lymphoid origin. The blood and bone marrow blasts from both patients at the time of leukemic transformation resembled lymphoblasts of ALL by morphological and histochemical evaluation and were typed as null cells by marker analysis. McCaffrey et al. (2) likewise found low levels of TdT in peripheral blood samples of two patients with lymphosarcoma-cell leukemia.

We have observed levels of TdT activity ranging from 0.05 to 1,000 nmol nucleotide incorporated/h per 10^8 cells, demonstrating the sensitivity of the analytical conditions over four orders of magnitude. Our assay appears to be somewhat more sensitive than assays used by others. We feel this is because sonication in the presence of 0.25 M phosphate buffer provides optimal extraction of the enzyme and the use of crude extract avoids fractionation losses. The initiator, poly d(pA)$_{\overline{50}}$, and [^{3}H]dGTP substrate minimize the effects of nucleases by forming a "nuclease-insensitive" product for assay. Cacodylate buffer is optimal for TdT but inhibits DNA polymerases α and β, minimizing the possibility of low level interference by these activities. Nucleotide at a concentration of 1 mM is required for maximal reaction velocity. In an attempt to make the assay practical and economical for large numbers of samples, 1 mM [^{3}H]dGTP at a spec act of 100 cpm/pmol is routinely used. Under these conditions, samples incorporating 1 nmol nucleotide/h per 10^8 cells (1 U) develop counts that are only two- to three-times background radioactivity, so samples assaying less than that cannot be reliably distinguished from zero activity. Increasing the specific activity of the nucleotide to 1,000 cpm/pmol decreases the detection limit to about 0.05 U/10^8 cells.

TdT activity was detectable at 0.07–1.61 U/10^8 cells in only 4 of the 11 peripheral blood samples from childhood controls and the remainder were <0.05 U/10^8 cells. Although the biological significance of these low values was not explored in this study, perhaps these levels reflect enzyme activity in the circulating hematopoietic stem cells described by Barr et al. (29). That is, it might be possible to assay for this stem cell activity in normal individuals by using other concentrative and separative procedures (30) not used in the present study.

The data presented indicate that limitations must be applied to clinical interpretation of blood and bone marrow TdT activity since enzyme levels of 13 and 15 U/10^8 cells were detected in two children with

morphologically normal specimens and a value of 12 U/10^8 cells was present in the bone marrow of a child with ITP. If current investigations aimed at identification of the TdT-positive cell in normal marrows are successful, they may resolve this problem and provide further insight into the biochemical basis of bone marrow lymphoid cell differentiation. At the present time, the clinical use of the enzyme should be restricted to differentiating leukemic cell types and classifying lymphoid malignancies. Although markedly elevated levels of the enzyme were present in ALL patients at diagnosis and relapse and low levels were present during disease remission, the data are not sufficient to suggest use of TdT determinations for monitoring disease activity or predicting relapse. Multiple variable analyses are required in monitoring disease activity and extensive studies will be required to assess the reliability of the TdT assay in comparison to careful morphological evaluations. Further studies at the cellular level by immunological methods (23, 31) may produce more distinctive results.

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