

Clinical Utility of Initial Terminal Deoxynucleotidyl Transferase Determinations in Childhood Acute Leukemias¹

David K. Kalwinsky,² William H. Weatherred, Gary V. Dahl, W. Paul Bowman, Susan L. Melvin, Mary Sue Coleman,³ and F. J. Bollum

Divisions of Hematology-Oncology [D. K. K., W. H. W., G. V. D., W. P. B.] and Immunology [S. L. M.], St. Jude Children's Research Hospital, Memphis, Tennessee 38101; Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky 40536 [M. S. C]; and Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20014 [F. J. B.]

ABSTRACT

Terminal deoxynucleotidyl transferase (TDT) activity was measured in bone marrow lymphoblasts obtained at diagnosis from 168 consecutive patients with childhood acute leukemia. Absolute concentrations of TDT were increased (≥ 20 units/ 10^6 blasts) in samples from 98 of 112 assessable patients with acute lymphocytic leukemia (ALL). The values ranged from < 1 to 1502 units/ 10^6 blasts with a median of 90 units contrasted with < 1 to 219 units (median, 2.6 units) in studies of children without leukemia. Results of an immunofluorescence assay were in good agreement with enzymatic detection of the polymerase. Among 115 patients with adequate marrow smears, 105 had TDT-positive blasts. By contrast, in most children with acute myelogenous leukemia, TDT activity was either undetectable or less than 10 units/ 10^6 blasts. Although the highest levels of TDT were found in blasts with the common ALL phenotype, quantitative determinations were not significantly related to the major immunological subtypes of ALL or to morphological features or periodic acid-Schiff reactivity of the lymphoblasts. The probability that a newly diagnosed case of leukemia would be ALL was 90% if TDT levels were greater than 20 units/ 10^6 blasts. We conclude that absolute concentrations of TDT, as determined in this study, are of little value in identifying subclasses of ALL. The immunofluorescence assay, which is much less expensive and easier to perform than the enzyme assay, should prove useful for confirming the diagnosis of ALL and for detecting extramedullary sites of leukemic infiltration.

INTRODUCTION

TDT,⁴ a mammalian DNA polymerase lacking template requirements, has been proposed as a biological marker for confirming the diagnosis of ALL, for aiding in its subclassification, and for monitoring leukemic activity during chemotherapy, remission, and relapse (4, 19). This enzyme was initially detected in high concentration in thymus tissue (3) and has since been found in a small but consistent percentage of normal marrow cells (11). After its discovery in the blasts of a child with ALL (23), more extensive screening of adult and childhood leukemias disclosed that an increased blast cell content of TDT is a characteristic feature of most patients with

this disease (9, 15, 16, 18, 22). In contrast, blasts from patients with AML have only sporadically been reported to contain detectable levels of the polymerase (15, 27).

In this study, we determined the absolute TDT content of bone marrow lymphoblasts from 168 consecutive children with untreated ALL or AML. Results were compared with morphological, cytochemical, and surface marker findings to assess the utility of this enzyme in identifying and subclassifying cases of ALL. In addition, a rapid immunofluorescence assay adapted to bone marrow smears was evaluated for its reliability and sensitivity in detecting TDT-positive leukemic cells in these same patients.

MATERIALS AND METHODS

Marrow samples from 168 consecutive children newly diagnosed as having either ALL (125 patients) or AML (43 patients) were assayed both quantitatively and by immunofluorescence for TDT activity. At our institution, ALL is identified on the basis of marrow replacement with leukemic cells that morphologically resemble lymphoblasts, whereas AML is distinguished by marrow infiltration with cells showing either myeloid or monocytic differentiation, as determined morphologically or cytochemically. Cytochemical stains, including PAS, peroxidase, Sudan black, naphthol AS-D chloroacetate esterase, and α -naphthyl acetate esterase, are routinely applied to all initial marrow smears. The final diagnosis in each case is reviewed by 3 experienced investigators. By convention, cases of stem cell or undifferentiated leukemia lacking any definitive cytochemical findings are designated as ALL. In addition, all leukemias are subclassified by morphological criteria of the French-American-British Cooperative Group (1) after independent review by 3 investigators (in this study, D. K. Kalwinsky, G. V. Dahl, and W. P. Bowman).

These investigations were performed with the approval of the Clinical Trials Committee of St. Jude Children's Research Hospital.

Sample Preparation. Bone marrow samples, 2 to 3 ml, were collected in EDTA (for TDT determinations) or in preservative-free heparin (for determination of surface markers) and enriched for blasts on Ficoll metrizoate cushions.

Quantitative Assay. Terminal transferase activity was measured under optimal conditions with oligodeoxyadenylic acid, with an average chain length of 50 residues, as the initiator and [³H]dGTP as the monomer (8). Initiator reagent was prepared in the laboratory of Dr. Mary Sue Coleman. All values are expressed as units per 10^6 blasts, where 1 unit equals 1 nmol of dGTP polymerized per hr at 35° . With this assay, TDT concentrations of less than 1 unit cannot be detected. In bone marrow samples from 198 children without leukemia, TDT

¹ Supported by Leukemia Program Project Grant CA 20180, by ALSAC, and by Grant CA 26391 (NCI).

² To whom requests for reprints should be addressed.

³ Recipient of Research Career Development Award CA 00494.

⁴ The abbreviations used are: TDT, terminal deoxynucleotidyl transferase; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; PAS, periodic acid-Schiff; E-rosettes, sheep erythrocyte rosettes.

Received December 29, 1980; accepted April 14, 1981.

values ranged from <1 to 219 units/10⁸ blasts with a median value of 2.6 units and a mean of 5.9 ± 19 (S.D.) units (19). For this study, any value of 20 units or more was considered increased (15). All quantitative assays were performed by Dr. Mary Sue Coleman at the University of Kentucky, using samples previously frozen at -70°.

Immunofluorescence Assay. In conjunction with quantitative determinations of TDT in cell extracts, methanol-fixed marrow smears were simultaneously processed for immunofluorescence studies of TDT localization. The indirect immunofluorescence assay has been reported in detail (4) and is based on detection of monovalent antibody prepared against purified bovine TDT. Marrow smears were examined within 5 days of sample aspiration by Zeiss epifluorescence microscopy. The optimal reagent concentrations were standardized by using the human lymphoid cell line 8402, which contains 325 units of TDT per 10⁸ cells (5). For each sample, a portion of the slide material was incubated with normal rabbit serum instead of rabbit anti-TDT to detect nonspecific fluorescence. Purified rabbit anti-TDT as well as fluorescein isothiocyanate-F(ab')₂-goat anti-rabbit IgG were prepared in the laboratory of Dr. F. J. Bollum.

Cell Surface Markers. E-rosette tests were standardized as reported previously (24). Fixed slides were prepared for morphological examination, and cells were considered to be E-rosette positive only if the rosette-forming cells were recognizable blasts. Conventional direct and indirect immunofluorescence methods (6) were used to assay cells for surface markers. Test results for any marker were judged as positive if 45% or more blasts had surface fluorescence. This criterion is derived from earlier studies by Melvin (25) in which a 45% lower limit was required for adequate segregation of lymphoblasts into subtypes. Rabbit antisera against a common ALL antigen (28), human Ia-like antigen (2), and antigens to human T-cells (24, 25) were prepared by standard methods. Antiimmunoglobulin reagents for detecting surface-bound antibody were purchased from Meloy Laboratories, Inc., Springfield, Va.

In this study, common ALL is defined as leukemia in which the blasts express both common ALL and Ia-like antigens on their surface. T-cell disease is identified by the presence of T-cell markers, either spontaneous E-rosette formation with sheep RBC or reactivity with antithymocyte serum. B-cell leukemia is defined by blasts having membrane-bound immunoglobulins, while undifferentiated ALL has only Ia-like antigen or lacks all of the above markers.

Cytochemical Studies. Marrow specimens were stained with PAS, peroxidase, Sudan black, and esterase according to standard methods (17, 29). PAS reactivity was considered positive when 20% or more of the blasts showed block or diffuse activity.

Statistical Analysis. Statistical comparison of TDT values with morphological, cytochemical, and surface marker results was performed with Student's *t* test and the *F* test. Natural logarithms of the TDT results were used in statistical comparisons of leukemia cell characteristics (Table 1) in order to normalize values around the mean. Cox's regression model (12) was used to express the probability that any given leukemic sample represented ALL based on TDT content alone.

RESULTS

ALL. Sufficient marrow cells were obtained from 112 of 125

patients with ALL for TDT determination. As shown in Chart 1a, TDT activity was increased in 98 samples (88%) with a median value of 90 units/10⁸ blasts; this represents a 30-fold enhancement over the median activity of TDT (2.6 units/10⁸ blasts) in nonleukemic pediatric marrow (19). The pattern of enzyme activity was strikingly heterogeneous, as reflected by a range of values from <1 to 1502 units/10⁸ blasts. In addition, 14 patients had TDT levels of less than 20 units, which is within 1 S.D. of the mean activity for children without leukemia.

Table 1 summarizes the statistical comparison of TDT levels with French-American-British Cooperative Group type, PAS reactivity, and surface marker studies. Quantitative determination of TDT did not aid in distinguishing L1 or L2 lymphoblasts or in identifying PAS-reactive leukemias. Although the highest TDT levels were found in cells with a common ALL phenotype, differences in the distribution of TDT activity among cases of common, T-cell, or undifferentiated ALL were not significant. Our 2 cases of B-cell ALL, both characterized by L3 morphology, did not have detectable TDT activity.

Table 1
Comparison of leukemia cell characteristics and TDT activity in children with ALL

	No. of patients	TDT activity (LN)	P
Morphology ^a			
L1	76	4.26 ± 1.10 ^b	0.07 ^c
L2	34	4.73 ± 1.31	
PAS reactivity			
Positive	47	4.50 ± 1.21	0.26 ^c
Negative	65	4.20 ± 1.17	
Surface markers			
Common	76	4.50 ± 1.16	0.82 ^d
T-cell	24	4.29 ± 1.15	
Undifferentiated	7	4.05 ± 1.27	
B-cell	2	ND ^e	

^a By French-American-British Cooperative Group criteria.
^b Mean ± S.D.
^c By Student's *t* test.
^d By *F* test.
^e ND, not detectable.

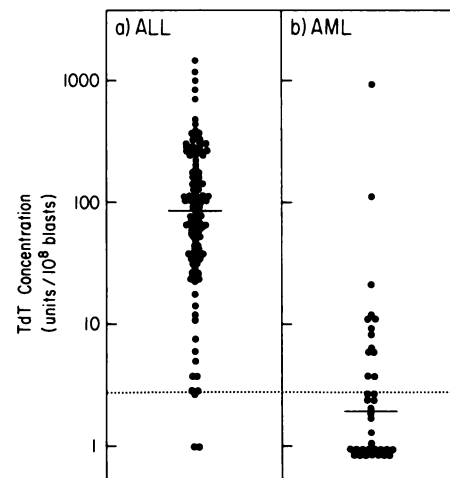
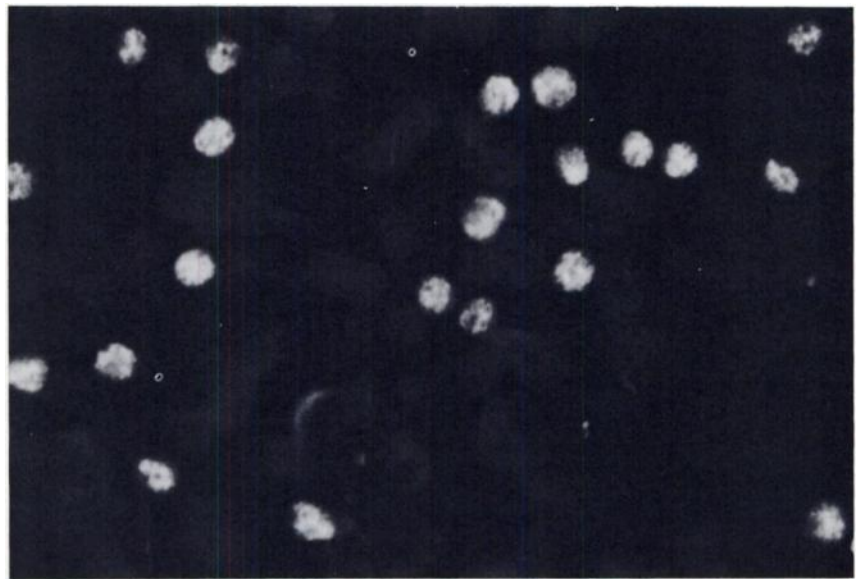


Chart 1. Terminal transferase activity in cell extracts from children with newly diagnosed ALL (n = 112) or AML (n = 37). The median activity (bar) for ALL cases is 90 units/10⁸ blasts and 1.9 units for AML cases. Lymphoblast samples were separated on a Ficoll gradient, and TDT activity was determined with oligodeoxyadenylic acid, with an average chain length of 50 residues, as the initiator and [³H]dGTP as the monomer (see "Materials and Methods"). One unit of activity equals 1 nmol of dGTP polymerized per hr at 35°. - - - -, median activity of 5.9 units for 198 children without leukemia (19).

Fig. 1. Typical immunofluorescence pattern of TDT-positive lymphoblasts in ALL patient marrow. Oil, $\times 480$.



The immunofluorescence assay for TDT was technically satisfactory in 115 ALL samples, and in 105 of these the blasts were clearly TDT positive. Transferase activity was localized within the nucleus of leukemic cells, as shown by phase microscopy (Fig. 1). In 95 ALL samples, virtually 100% of the blasts stained strongly for TDT, whereas in 10 others only a fraction (30 to 60%) of the blasts were TDT positive. Ten patients with technically satisfactory immunofluorescent preparations lacked detectable enzyme activity. In 2 of these cases, both B-cell ALL, neither the immunofluorescence nor the enzyme assay disclosed TDT activity; in each of the remaining 8 cases, the enzyme was quantitatively detectable over a range of 11 to 489 units/ 10^8 blasts.

AML. Quantitative TDT determinations were made for 37 of 43 consecutive patients with AML. Nearly one-half of the samples lacked detectable TDT activity; the median value for all samples was 1.9 units/ 10^8 blasts (Chart 1b). In 3 cases, the TDT values were within the range seen for ALL samples, but the blasts were unequivocally nonlymphocytic in origin. These TDT-positive samples contained either Auer rods or cytochemical evidence of myeloid or myelomonocytic differentiation (Table 2). The immunofluorescence assay, performed on all 43 AML patients, failed to detect polymerase in samples with less than 10 units of activity. However, the 3 AML samples with quantitatively elevated enzyme did show a strong nuclear staining pattern indistinguishable from that seen in ALL.

Statistical Function. Since most samples from patients with ALL had increased TDT values whereas AML marrows had low or undetectable activity, we were able to calculate a function to express the probability that any new patient with acute leukemia has ALL based on the initial TDT value alone. With Cox's regression model, p or the probability of being ALL is expressed as

$$\log p/1 - p = \alpha + \beta \ln \text{TDT}$$

where $\alpha = -2.075$ and $\beta = 1.204$; this function is shown in Chart 2. From Chart 2, it is apparent that patients with TDT values of 20 units or more at diagnosis ($\ln \text{TDT}$, 3) have about a 90% probability of being ALL, whereas in samples without detectable TDT activity the probability that new cases will be

Table 2
Cytochemical and morphological features in 3 cases of TDT-positive AML

Morphology ^a	Cytochemical type ^b	TDT activity (units/ 10^8 cells)
M1	SB, PX	948.6
M2	SB, PX, NCE	21.1
M4 (Auer rods)	PX, ANE	115.8

^a By French-American-British Cooperative Group criteria.

^b Sudan black; PX peroxidase; NCE, naphthol AS-D chloroacetate; ANE, α -naphthyl acetate esterase.

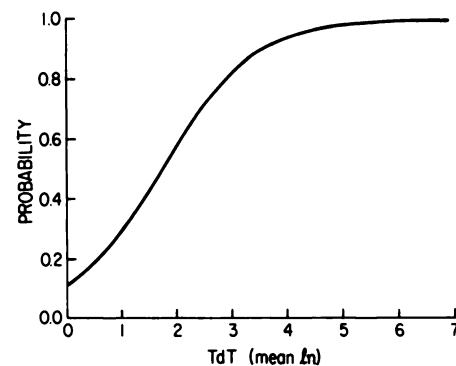


Chart 2. Probability that any sample of leukemic cells represents ALL based on TDT content alone.

lymphocytic leukemia is only 8%.

DISCUSSION

This study of bone marrow lymphoblasts from a large population of consecutive pediatric patients demonstrates the close association of TDT activity with childhood ALL. Polymerase activity was detected in each of our ALL cases if both an immunofluorescence and an enzyme assay were used.

Some investigators have suggested that, in patients with ALL, quantitative measurements of TDT in blast cells may correspond to the degree of surface marker maturation (22) and hence complement surface marker typing to define biological subtypes of ALL (10, 22). This impression comes from studies with relatively small numbers of inconsecutive patients,

in which TDT activity was greatest in non-T and non-B ALL, intermediate in T-cell ALL, and lowest in B-cell cases. As shown in Table 1, absolute concentrations of TDT did not contribute to improved identification of subclasses of ALL in our study, nor did they correlate with morphological findings or cytochemical stain patterns, aside from confirming the lack of this polymerase in B-cell or L3-type ALL (10, 15). In particular, patients with T-cell disease did not, on the average, have lower TDT activity than did patients with common ALL. Treatment duration is at present too short to determine if the quantitative assessment of TDT has any correlation with clinical outcome.

Our immunofluorescence studies demonstrated the nuclear localization of TDT in leukemic lymphoblasts, in contrast to the cytoplasmic distribution of the enzyme in thymocytes (14). The immunofluorescence assay was not quantitative, since marrow smears from samples containing 20 to 1500 units of TDT activity were stained equally intensely by the immunofluorescence conjugate. Ten % of the ALL cases had TDT by enzyme assay but lacked detectable transferase by immunofluorescence. This apparent discrepancy between the 2 assays applied to the same samples has been previously noted (19) and may reflect an altered antigenic form(s) of TDT in malignant cells, as suggested by the presence of a distinctive molecular variant of the transferase in human leukemia cell lines (5, 13).

The finding of a 10% incidence of TDT positivity in consecutive pediatric cases of AML is supported by reports of TDT-positive AML in both children and adults (4, 15, 27). There were no unusual morphological, cytochemical, or surface marker features in our positive cases, and none represented unrecognized chronic myelogenous leukemia in blastic crisis with myelocytic morphology (*i.e.*, all cases lacked the Philadelphia chromosome). Vincristine and prednisone were not included in the treatment regimens for this series of AML patients. Remission durations in these cases with TDT-positive blasts (2, 10, and 17+ months, respectively) have been similar to our cases without detectable polymerase (mean duration of remission of 10 months). A larger number of TDT-positive AML patients should be studied for longer periods to determine if this phenotype represents a clinically significant subgroup.

Sarin (26) and others (4) have proposed that an immunofluorescence assay for TDT be used to identify residual marrow lymphoblasts signifying early ALL relapse. Although this is technically feasible, such an application would be complicated by the presence of TDT-positive cells with an Ia/common ALL phenotype in 2 to 10% of nucleated cells from normal pediatric marrows (21) and by the substantial number of nonleukemic conditions (*e.g.*, marrow regeneration, idiopathic thrombocytopenic purpura, neuroblastoma) known to be associated with positive TDT immunofluorescence in marrow samples (19, 20). Moreover, by immunofluorescence we have been able to detect up to 11% TDT-positive cells in 49 nonleukemic "control" marrows from solid tumor patients at this institution.⁵ This background of TDT-positive marrow cells in nonleukemic marrows seriously limits the value of the immunofluorescence assay for detecting early leukemic relapse, as was recently demonstrated by Janossy *et al.* (20) using dual immunofluorescent markers. Alternatively, immunofluorescence might be used to improve the accuracy of diagnosis of extramedullary leukemia, in both the testis and the spinal fluid, if applied to

cyto centrifuge slides of spinal fluids or to touch preparations of testicular wedge biopsies (7).

We conclude that the immunofluorescence assay for terminal transferase is an easily performed, rapid, and reliable test for confirming the diagnosis of ALL. Because of its lack of absolute specificity, however, the assay should not be used alone but should be combined with morphological determinations, surface typing, and cytochemical staining in the diagnosis of new leukemias. Since the quantitative test for TDT did not aid in the classification of cases of ALL and is costly and complicated to perform, it is no longer being used routinely as a diagnostic tool at our institution. Studies to evaluate the diagnostic potential of this assay for extramedullary leukemia, using spinal fluid and testicular biopsies, are under way.

REFERENCES

- Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A. G., Grabmon, H. R., and Sultan, C. Proposals for classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br. J. Haematol.*, 33: 451-458, 1976.
- Billing, R., Rafizadeh, B., Drew, I., Hartman, G., Cale, R., and Terasaki, P. Human B-lymphocyte antigens expressed by lymphocytic and myelocytic leukemia cells. Detection by rabbit antisera. *J. Exp. Med.*, 144: 167-178, 1976.
- Bollum, F. J. Calf thymus polymerase. *J. Biol. Chem.*, 235: 2399-2403, 1960.
- Bollum, F. J. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. *Blood*, 54: 1203-1215, 1979.
- Bollum, F. J., and Brown, M. A high molecular weight form of terminal deoxynucleotidyl transferase. *Nature (Lond.)*, 278: 191-192, 1979.
- Borella, L., and Sen, L. The distribution of lymphocytes with T- and B-cell surface markers in human bone marrow. *J. Immunol.*, 112: 836-843, 1974.
- Bradstock, K. F., Papageorgiou, E. D., Janossy, G., Huffband, A. V., Willoughby, M. L., Roberts, P. D., and Bollum, F. J. Detection of leukemic lymphoblasts in CSF by immunofluorescence for terminal transferase. *Lancet*, 1: 1144, 1980.
- Coleman, M. S. Terminal deoxynucleotidyl transferase: characterization of extraction and assay conditions from human and calf tissue. *Arch. Biochem. Biophys.*, 182: 525-532, 1977.
- Coleman, M. S., Greenwood, M. F., Hutton, J. J., Bollum, F. J., Lampkin, B., and Holland, P. Serial observations on terminal deoxynucleotidyl transferase activity and lymphoblast surface markers in acute lymphoblastic leukemia. *Cancer Res.*, 36: 120-127, 1976.
- Coleman, M. S., Greenwood, M. F., Hutton, J. J., Holland, P., Lampkin, B., Krill, C., and Kastelic, J. E. Adenine deaminase, terminal deoxynucleotidyl transferase (TdT), and cell surface markers in childhood acute leukemia. *Blood*, 52: 1125-1131, 1978.
- Coleman, M. S., Hutton, J. J., De Simone, P., and Bollum, F. J. Terminal deoxynucleotidyl transferase in human leukemia. *Proc. Natl. Acad. Sci. U. S. A.*, 71: 4404-4408, 1974.
- Cox, D. R. *The Analysis of Binary Data*, pp. 1-42. London: Methuen & Co., Ltd., 1970.
- Deibel, M. R., and Coleman, M. S. Purification of a high molecular weight human terminal deoxynucleotidyl transferase. *J. Biol. Chem.*, 254: 8634-8640, 1979.
- Goldschneider, I., Gregoire, K. E., Barton, R. W., and Bollum, F. J. Demonstration of terminal deoxynucleotidyl transferase in thymocytes by immunofluorescence. *Proc. Natl. Acad. Sci. U. S. A.*, 74: 734-738, 1977.
- Gordon, D. S., Hutton, J. J., Smalley, R. V., Meyer, L. M., and Volger, W. R. Terminal deoxynucleotidyl transferase (TdT) cytochemistry, and membrane receptors in adult acute leukemia. *Blood*, 52: 1079-1088, 1978.
- Greenwood, M. F., Coleman, M. S., Hutton, J. J., Lampkin, B., Keill, C., Bollum, F. J., and Holland, P. Terminal deoxynucleotidyl transferase distribution in neoplastic and hematopoietic cells. *J. Clin. Invest.*, 59: 889-899, 1977.
- Hayhoe, F. G. J., and Quaglino, D. *Haematological Cytochemistry*. Edinburgh: Churchill Livingstone, 1980.
- Hoffbrand, A. V., Ganeshaguru, K., Janossy, G., Greaves, M. F., Catovsky, D., and Woodruff, R. K. Terminal deoxynucleotidyl-transferase levels and membrane phenotypes in diagnosis of acute leukaemia. *Lancet*, 2: 520-523, 1977.
- Hutton, J. J., Coleman, M. S., Keneklis, T. P., and Bollum, F. J. Terminal deoxynucleotidyl transferase as a tumor cell marker in leukemia and lymphoma, results from 1,000 patients. *In: E. Mihich and R. Baserga (eds.), Advances in Medical Oncology, Research and Education, Vol. 4*, pp. 163-174. Elmsford, N. Y.: Pergamon Press, Inc., 1979.
- Janossy, G., Bollum, F. J., Bradstock, K. F., and Ashley, J. Cellular pheno-

⁵ D. K. Kalwinsky and W. H. Weathered, unpublished observations.

- types of normal and leukemic hematopoietic cells determined by analysis with selected antibody combinations. *Blood*, 56: 430-441, 1980.
21. Janossy, G., Bollum, F. J., Bradstock, K. F., McMichael, A., Rapson, N., and Greaves, M. Terminal transferase of positive human bone marrow cells exhibiting the antigenic phenotype of common acute lymphoblastic leukemia. *J. Immunol.*, 125: 1525-1529, 1979.
 22. Janossy, G., Hoffbrand, A. V., Greaves, M. F., Ganeshaguru, K., Pain, C., Bradstock, K. F., Prentice, H. G., Kay, H. E. M., and Lister, T. A. Terminal transferase enzyme assay and immunological membrane markers in the diagnosis of leukemia: a multiparameter analysis of 300 cases. *Br. J. Haematol.*, 44: 221-234, 1980.
 23. McCaffrey, R., Smoler, D. F., and Baltimore, D. Terminal deoxynucleotidyl transferase in a case of childhood acute lymphoblastic leukemia. *Proc. Natl. Acad. Sci. U. S. A.*, 70: 521-525, 1973.
 24. Melvin, S. L. Comparison of techniques for detecting T-cell acute lymphocytic leukemia. *Blood*, 54: 210-215, 1979.
 25. Melvin, S. L. Immunologic definition of leukemic cell surface phenotypes. *Cancer Res.*, in press, 1981.
 26. Sarin, P. S. Terminal transferase as a biological marker for human leukemia. *In: R. C. Gallo (ed.), Recent Advances in Cancer Research: Cell Biology, Molecular Biology, and Tumor Virology*, Vol. 1, pp. 131-138. West Palm Beach, Fla.: CRC Press, 1977.
 27. Srivastava, B. I. S., Khan, S. A., and Henderson, E. S. High terminal deoxynucleotidyl transferase activity in acute myelogenous leukemia. *Cancer Res.*, 36: 3847-3850, 1976.
 28. Veit, B. C., Melvin, S. L., and Bowman, W. P. Identification of a leukemia-associated antigen of human acute lymphocytic leukemia. *J. Natl. Cancer Inst.*, 64: 1321-1328, 1980.
 29. Yam, L. T., Li, C. Y., and Crosly, W. H. Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol.*, 55: 283-290, 1971.