ANALYSIS OF RAT HEMOPOIETIC CELLS ON THE FLUORESCENCE-ACTIVATED CELL SORTER

II. Isolation of Terminal Deoxynucleotidyl Transferase-positive Cells*

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Terminal deoxynucleotidyl transferase (TdT)[1] is an intracellular enzyme that can catalyze the polymerization of any 3'-OH-terminated segment of DNA without template direction (1). In rat and mouse, TdT is present in the majority of cortical thymocytes and in a minority of nucleated cells from bone marrow and prepubertal spleen, liver, and blood (2–6) (R. Sasaki, F. J. Bollum, and I. Goldschneider. Transient populations of terminal deoxynucleotidyl transferase-positive cells in rats and mice. Manuscript in preparation.). It is also present in most cases of acute lymphoblastic leukemia (ALL) in man and mouse, and in some cases of chronic myelogenous leukemia in blast crisis (7, 8). Because of its restricted distribution and unusual biochemical properties, it has been postulated that TdT may function as a somatic mutagen during the early stages of lymphocyte differentiation (9, 10).

There is strong evidence that many TdT^+ hemopoietic cells are thymocyte progenitors (11). Approximately 50% of TdT^+ cells in normal mouse bone marrow can be induced to express Thy-1 antigen by incubation in vitro with thymopoietin (12); and >75% of TdT^+ cells in bone marrow and spleen from normal and congenitally athymic (nu/nu) mice can be induced to express Ly-1,2,3 antigens by incubation with thymosin.[2] TdT also has been detected in most cases of human pre-B cell leukemia (i.e., cytoplasmic Ig^+, surface Ig^-) (13, 14) and in a small percentage of pre-B cells in normal human (15) and mouse (16) bone marrow. This suggests that some TdT^+ cells may be B cell progenitors or lymphoid stem cells.

[1] *Abbreviations used in this paper* ALL, acute lymphoblastic leukemia; CFC, in vitro colony-forming cells, CFU-S, in vivo spleen colony-forming unit(s), EO, eosinophil, FACS, fluorescence-activated cell sorter, GM, granulocyte-macrophage; MEG, megakaryocyte, PHSC, pluripotent hemopoietic stem cell(s), TdT, terminal deoxynucleotidyl transferase

[2] Goldschneider, I., A. Ahmed, F J Bollum, and A L Goldstein Induction of terminal deoxynucleotidyl transferase and Lyt antigens with thymosin identification of multiple subsets of prothymocytes in mouse bone marrow and spleen cells Manuscript submitted for publication

Studies to test these hypotheses directly would be aided by the availability of highly enriched populations of viable TdT$^+$ cells, especially if such populations were devoid of pluripotent hemopoietic stem cells. This is accomplished in the present study, in which the fluorescence-activated cell sorter (FACS) (17) is used to isolate TdT$^+$ cells from normal rat bone marrow.

Materials and Methods

All materials and methods used in this study are described elsewhere (18), except for the following.

Immunofluorescence Assay for TdT. An IgG fraction of rabbit antiserum to homogenous calf TdT was rendered highly specific by elution from a TdT-immunoadsorbant column as described previously (3, 19). The anti-TdT was used at a concentration of 200 μg of protein/ml of Eisen's balanced salt solution. Fluorescein-conjugated goat IgG against rabbit IgG was obtained from N. L. Cappel Laboratories Inc., Cochranville, Pa.

Cell smears were prepared in a cytocentrifuge (Cytospin; Shandon Southern Instruments Inc., Sewickley, Pa.), fixed for 5 min in cold, absolute methanol, and processed for indirect immunofluorescence as described previously (3). Percentages of TdT$^+$ cells were based on examination of 1,000 nucleated cells/sample.

Results

Distribution of TdT$^+$ Cells by FACS Analysis

LOW-ANGLE LIGHT SCATTER. Approximately 97% of TdT$^+$ cells from rat bone marrow were present between the peaks formed by lymphocytes (peak II) and myeloid cells (peak III); 81% of the TdT$^+$ cells were concentrated in a 30-channel window at the base of the valley between these two peaks (Fig. 1, fraction B; the entire light scatter distribution comprised 256 channels). Collection of this fraction, which contained ~14% of the total nucleated bone marrow cells, resulted in a mean 4.9-fold enrichment in TdT$^+$ cells (Table I). Any substantial enlargement of this window to the left or right resulted in slightly greater recovery of total TdT$^+$ cells but in significantly lesser enrichment. Therefore, all further purification was restricted to the cells in fraction B.

RELATIVE FLUORESCENCE INTENSITY FOR THY-1 ANTIGEN. As reported previously (3), all TdT$^+$ cells in rat bone marrow are Thy-1$^+$. Results in Fig. 2 and Table II show
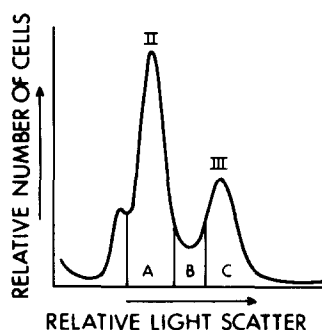

Fig 1   Relative size distribution of rat bone marrow cells as determined by low-angle light scatter on the FACS. Roman numerals designate lymphoid (II) and myeloid (III) cell peaks (18). Erythrocytes (formerly peak I) were lysed with 0.168 M NH$_4$Cl Vertical threshold markers delineate cell fractions (capital letters) that were separated In this figure, fraction B contained 12 8% of total nucleated cells and 81 7% of total TdT$^+$ cells

TABLE I

Distribution of TdT$^+$ Cells in Rat Bone Marrow According to Relative Low-Angle Light Scatter

Fraction (%)*	Percentage of TdT$^+$ cells/fraction‡	Percentage of total TdT$^+$ cells	Enrichment§
	%	%	
I (<1)	0 0	0 0	<1
II$_L$ (30 ± 6 5)	0 0	0 0	<1
II$_R$ (24 ± 3 6)	5 0 ± 0 8	52 1	2 3
III$_L$ (22 ± 3 6)	4 9 ± 0.8	44 9	2 2
III$_R$ (24 ± 3 9)	0 3 ± 0 1	3 0	<1
A (50 3 ± 1 7)	0 7 ± 0 2	16 6	<1
B (14 3 ± 1 5)	10 8 ± 1 7	73 2	4 9
C (35 4 ± 2 5)	0 6 ± 0 1	10 1	<1
Unfractionated‖	2 2 ± 0 5	—	—

* See Fig 1 for details L, left half of peak, R, right half of peak Numbers in parentheses refer to percentage of total nucleated cells
‡ Determined by immunofluorescence for TdT Mean ± SD of four to six experiments
§ Number of times greater than value in unfractionated bone marrow cells
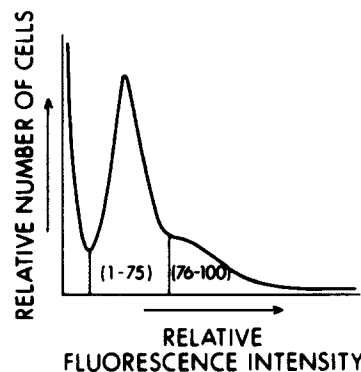‖ Not processed on the FACS

FIG 2   Relative fluorescence distribution of rat bone marrow cells incubated with rabbit fluorescein isothiocyanate-conjugated F(ab')$_2$ anti-Thy-1 serum FACS analysis of cells in fraction B (Fig 1) The numbers in parentheses indicate percentile of fluorescent cells in delineated fractions Note that the cells in the upper 25th percentile of Thy-1$^+$ cells form a distinct shoulder to the right of the main peak In this figure, 84 9% of the cells in the upper 25th percentile were TdT$^+$

that 91% of the TdT$^+$ cells in fraction B are in the upper 50th percentile for Thy-1 fluorescence, and that 78% are in the upper 25th percentile. Collection of this latter fraction yielded cell suspensions that contained a mean of 84.5% TdT$^+$ cells (Fig. 3), which represents a 45-fold enrichment over levels in unfractionated bone marrow

In addition to TdT$^+$ cells, pluripotent hemopoietic stem cells (PHSC) were enriched ~52-fold by the above fractionation procedure. However, PHSC are restricted to the upper 10th percentile of Thy-1$^+$ cells (18). Hence, by collecting only those cells in the 76-90th percentile for Thy-1 fluorescence, suspensions were obtained that contained a mean of 87% TdT$^+$ cells but <0.2% in vivo spleen colony-forming units (CFU-S) (assuming a seeding efficiency of 0 01)

TABLE II

Distribution of TdT$^+$ Cells in Rat Bone Marrow According to Relative Fluorescence Intensity for Thy-1 Antigen

Thy-1$^+$ cells percentile*	Percentage of TdT$^+$ cells/fraction‡	Percentage of total TdT$^+$ cells§	Enrichment‖
	%	%	
91–100	83 2 ± 6.1	30 3	43 8
76–90	86.6 ± 5 9	47.4	45 6
76–100	84 5 ± 5.0	77.7	44 5
1–75	8.1 ± 2.7	22 3	4 3
51–100	56 1 ± 9 1	91 2	29 5
1–50	5 4 ± 2.7	8 8	2 8
Total Thy-1$^+$	31.4 ± 5 8	100 0	16 5
Total Thy-1$^-$	0 0	0 0	<1
Unfractionated¶	1.9 ± 0 5	—	—

* Cells in fraction B (Fig 1 and Table I) were sorted into Thy-1$^+$ (fluorescent) and Thy-1$^-$ (nonfluorescent) populations on the FACS. The Thy-1$^+$ cells were sorted further according to relative fluorescence intensity (Fig 2)
‡ Determined by immunofluorescence for TdT   Mean ± SD of four to six experiments
§ Fraction B
‖ Number of times greater than value in unfractionated bone marrow
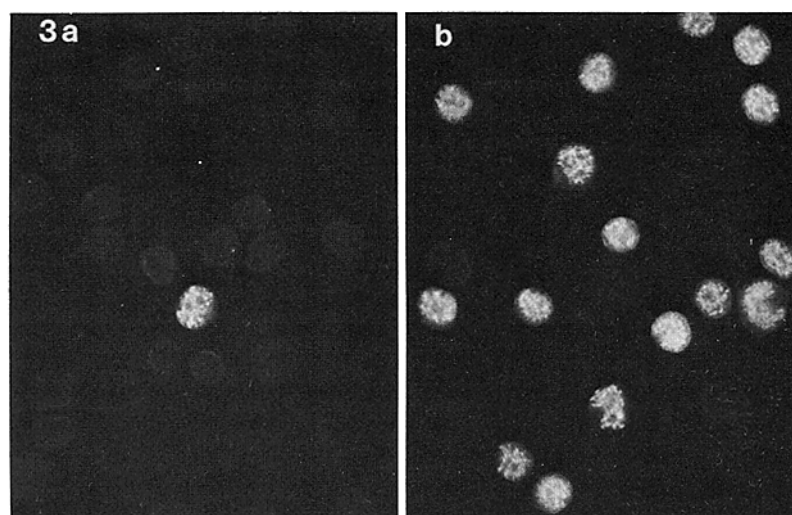¶ Not processed on the FACS

FIG. 3   Smears of FACS-separated rat bone marrow cells developed for immunofluorescence with antibodies to TdT   The unfractionated bone marrow contained 1 7% TdT$^+$ cells. (a) Cells from fraction B, 1–75th percentile for Thy-1 fluorescence   Note single TdT$^+$ cell (nuclear fluorescence) and numerous TdT$^-$ cells (appear as pale grey ghosts) 3 9% of cells in this fraction were TdT$^+$ (b) Cells from fraction B, 76–100th percentile for Thy-1 fluorescence 81% of cells in this fraction were TdT$^+$ × 560

Pretreatment of the donor rats with cortisone caused a 96% decrease of TdT$^+$ cells in the 76–100th percentile of Thy-1$^+$ cells in fraction B.

Morphology of TdT$^+$ Cells

TdT$^+$ cells have a characteristic appearance by light microscopy (Fig. 4). The cells superficially resemble large lymphocytes, but have a slightly eccentric, sharply indented, leptochromatic nucleus, multiple nucleoli, a prominent Golgi zone, and moderately abundant cytoplasm with a prominent basophilic rim. The mean diameter of TdT$^+$ cells in fraction B (upper 25th percentile of Thy-1$^+$ cells) was 10.4 ± 1.8 μm.

Most of the TdT$^-$ cells in the 91–100th percentile of Thy-1$^+$ cells in fraction B were large undifferentiated mononuclear cells that had the morphological characteristics of candidate PHSC (18). The percentage of these cells increased from 11 to 56 after treatment with cortisone. Almost all of the TdT$^-$ cells in the 76–90th percentile of Thy-1$^+$ cells in fraction B were polychromatophilic erythroblasts.

The Thy-1$^+$ cells in the lower 75th percentile of fraction B were a mixture of medium size lymphocytes (73%) and erythroblasts (27%).

The ultrastructural properties of candidate TdT$^+$ cells are shown in Fig. 5.

Discussion

Most schemes of lymphopoiesis envision the existence of discrete populations of lymphoid stem and/or progenitor cells. However, attempts to functionally demonstrate these cells have been complicated by the presence of PHSC. This problem has been partially obviated by the study of Abramson et al. (20), who described chromosomally marked clones of T cell progenitors. However, these authors were unable to demonstrate clones of B cell progenitors or multipotent lymphoid stem cells, thus prompting the speculation that PHSC may subserve these functions. Other authors have noted that the lymphoid and myeloid-erythroid cell series diverge early in hemopoiesis (21–23), further emphasizing the developmental proximity of primitive lymphocyte precursors to PHSC. Therefore, in attempting to identify TdT$^+$ cells as lymphocyte progenitors, it is essential to demonstrate that they are not PHSC or myeloid-erythroid cell precursors.

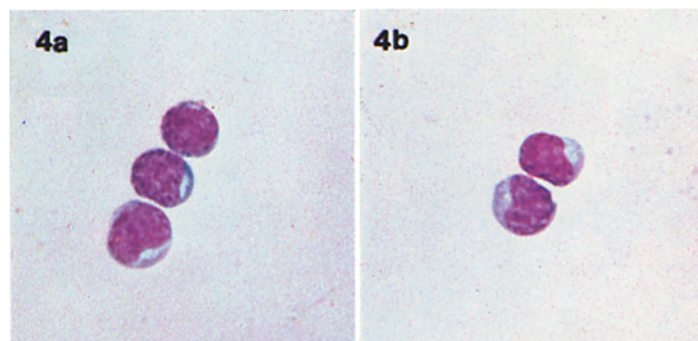As shown in Fig. 6, TdT$^+$ cells can be completely separated from granulocyte-

Fig 4   Candidate TdT$^+$ cells from rat bone marrow. May-Grunwald-Giemsa stain (a and b) Representative cells from suspensions containing 87% and 92% TdT$^+$ cells, respectively Note rim of basophilic cytoplasm, prominent Golgi zone, and indented, moderately leptochromatic nucleus Nucleoli are not well visualized in these photographs but were typically present (Fig 5)   × 775
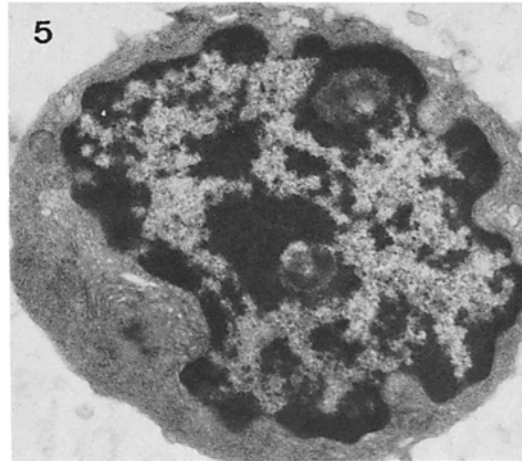
Fig 5   Electron micrograph of candidate TdT⁺ cell   The typical features of the cells in this population are their generally leptochromatic nuclei with prominent nucleoli, relatively abundant cytoplasm that contains numerous monoribosomes, a well developed Golgi zone and adjacent centriole, and some mitochondria   Other organelles are sparse or absent. × 7,000
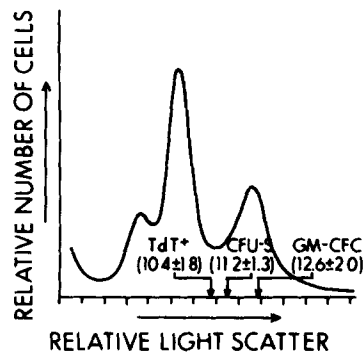

Fig. 6.   Relative size distribution of lymphohemopoietic precursor cells from rat bone marrow as determined by low-angle light scatter on the FACS. The arrows designate the position of TdT⁺ cells, CFU-S, and GM-CFC relative to each other and to total nucleated bone marrow cells (reference curve). Mean diameters (μm) ± SD were determined on cytocentrifuge smears of enriched fractions as described in this paper and elsewhere (18) The distance between the markings on the abscissa represent ∼1 μm.

macrophage (GM) in vitro colony-forming cells (CFC) (GM-CFC) on the basis of relative light scatter, TdT⁺ cells being much smaller than GM-CFC (18). Eosinophil (EO) and megakaryocyte (MEG) progenitors (EO-CFC and MEG-CFC) also are larger than TdT⁺ cells (I. Goldschneider. Unpublished observations.). Although the relationship between TdT⁺ cells and erythrocyte progenitor cells (erythroid CFU and erythroid burst-forming unit) was not formally tested, other studies have shown that erythrocyte progenitor cells are larger than PHSC (24), and, hence, they almost certainly are larger than TdT⁺ cells (see below).

TdT⁺ cells and PHSC overlap considerably with respect to relative size and fluorescence intensity for Thy-1 antigen (Fig. 6) (18). However, TdT⁺ cells are slightly smaller on average (mean diameter 10.4 μm) than are PHSC (mean diameter 11.2

μm) and have a broader representation among Thy-1$^+$ cells (upper 25th percentile vs. upper 10th percentile). Therefore, it is possible to separate about one-half of the TdT$^+$ cells in fraction B from PHSC on the basis of relative fluorescence intensity for Thy-1 antigen. That the remainder of the TdT$^+$ cells (upper 10th percentile for Thy-1 fluorescence) are also distinct from PHSC is shown by their differential sensitivity to cortisone, TdT$^+$ cells being sensitive and PHSC being resistant (18). The fact that PHSC candidates in fraction B increased approximately fivefold after cortisone treatment indicates that cortisone acts to physically deplete the population of TdT$^+$ cells, rather than simply to interfere with the biosynthesis of TdT.

The fractionation procedure that we have devised for isolating TdT$^+$ cells is not ideal, inasmuch as only 34% of total TdT$^+$ cells are recovered. Nonetheless, it does meet the major objective of providing a highly enriched population of viable TdT$^+$ cells virtually free from PHSC and myeloid progenitor cells. This should make it possible to study the ability of TdT$^+$ cells to generate T and/or B cells in adoptive transfer systems. If, as seems likely, TdT$^+$ cells are lymphocyte progenitors and/or lymphoid stem cells, the ability to culture them in vitro (25) (J. Hayashi, I. Goldschneider, and F. J. Bollum. A selective culture system for generating terminal deoxynucleotidyl transferase-positive cells *in vitro*. Manuscript in preparation.) and the availability of a variety of T and B cell-inducing agents may permit a detailed analysis of the early stages of lymphocyte differentiation in vitro.

A second area in which the availability of highly enriched populations of TdT$^+$ cells may be useful is in the study of the pathogenesis of ALL. Most cases of ALL in human beings and mice are TdT$^+$ (7, 8). However, it is not clear at what stages in the development of TdT$^+$ cells neoplastic transformation occurs. Janossy et al. (15) have reported that TdT$^+$ cells in nonleukemic human bone marrow bear ALL-associated antigens, thus suggesting that they may be precursors of the common type (null cell, non-T, and non-B) of ALL. By using Gross murine leukemia virus to induce lymphocytic leukemia in rats, we have shown that the neoplastic TdT$^+$ cells in thymus and blood contain a variant lactic dehydrogenase isoenzyme that normally is present in bone marrow but not in thymus.[3] Similarly, the levels of adenosine deaminase and purine nucleoside phosphorylase in the leukemic cells are characteristic of those in prethymic cells. Thus, it is possible that the target of neoplastic transformation is the TdT$^+$ bone marrow cell or its precursor. Purification of TdT$^+$ bone marrow cells during the latent period of leukemogenesis may provide direct evidence in support of this hypothesis.

Lastly, results of preliminary experiments indicate that the enriched population of TdT$^+$ cells can be used to raise specific antisera to surface antigens on TdT$^+$ cells These antisera should be useful in further purifying TdT$^+$ cells on the FACS and in tracing the development of TdT$^+$ cells. Indeed, one antiserum has been shown to react selectively with TdT$^+$ bone marrow cells and subcapsular thymocytes, thus suggesting that these two cell populations may have a precursor-product relationship (I. Goldschneider. Unpublished observation.).

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[3] Barton, R W, and I. Goldschneider Evidence for the hemopoietic origin of Gross virus induced lymphocytic leukemia in rats Description of an LDH isoenzyme variant in leukemic cells and normal bone marrow Manuscript submitted for publication

Summary

A method is described by which highly enriched populations of viable terminal deoxynucleotidyl transferase-positive (TdT$^+$) cells can be isolated from rat bone marrow by use of the fluorescence-activated cell sorter. Such cells have been postulated to be progenitors of thymocytes and, possibly, of B lymphocytes, and may serve as the targets of neoplastic transformation in acute lymphoblastic leukemia. The separation procedure is based on differences in relative low-angle light scatter and relative fluorescence intensity for Thy-1 antigen between TdT$^+$ cells and other lymphohemopoietic cell populations in bone marrow. Simultaneous sorting of bone marrow cells according to these two parameters resulted in a mean 87% purification of TdT$^+$ cells. The morphological characteristics of the isolated TdT$^+$ cells are described at the light and electron microscopic levels.

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