IDENTIFICATION OF THYMOCYTE PROGENITORS IN HEMOPOIETIC TISSUES OF THE RAT

II. Enrichment of Functional Prothymocytes on the Fluorescence-activated Cell Sorter*

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The thymus of irradiated animals can be repopulated by the progeny of precursors from hemopoietic tissues (1–5).[1] Although the existence of these functional prothymocytes has long been recognized, only modest progress has been made in identifying and isolating them. In the mouse, functional thymocyte progenitors are part of the "null" (non-T, non-B) cell compartment of bone marrow and spleen, and enrichments of 10- to 30-fold, respectively, have been obtained through depletion procedures in conjunction with density gradient centrifugation (6). Partial separation of prothymocytes and pluripotent hemopoietic stem cells (CFU-S)[2] has been observed using density gradient centrifugation or velocity sedimentation (7, 8).

The mouse bone marrow cell fractions that are enriched in prothymocyte activity have been found to be comparably enriched in terminal deoxynucleotidyl transferase (TdT) activity (6). Moreover, TdT-positive cells have been shown to express T cell surface antigens after brief in vitro induction with thymosin (9) and thymopoietin (10). Hence, they have been postulated to be the most likely candidates for prothymocytes in bone marrow.

Prothymocyte activity has also been found in adult spleen, which lacks TdT-positive cells (5);[1] and some TdT-negative cells in bone marrow and spleen can be induced to express T cell differentiation antigens (9). This has prompted the speculation that at least two subsets of prothymocytes exist in the mouse.

In the rat, unlike the mouse, the Thy-1.1 antigen is readily detectable on a variety of immature lymphohemopoietic cells in bone marrow, spleen, and fetal liver (11–14). By using multiple parameter analysis of relative cell size (low-angle light scatter), relative fluorescence intensity for Thy-1 antigen, and relative sensitivity to cortisone to resolve subsets of rat bone marrow cells on the fluorescence-activated cell sorter (FACS), we have developed protocols for separating and markedly enriching pluri-

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[1] Greiner, D. L., I. Goldschneider, and D. M. Lubaroff. Identification of thymocyte progenitors in hemopoietic tissues of the rat. I. A quantitative assay system for thymocyte regeneration. Manuscript submitted for publication.

[2] *Abbreviations used in this paper:* ART, antigen of the rat thymus; CFU-S, in vivo spleen colony-forming unit; FACS, fluorescence-activated cell sorter; TdT, terminal deoxynucleotidyl transferase.

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potent hemopoietic stem cells, myeloid progenitor cells, and TdT-positive cells (15, 16). We have also developed a thymocyte regeneration assay system to quantify relative prothymocyte activities in cell suspensions from rat bone marrow and spleen.[1] This assay uses antiserum to the antigen of the rat thymus (ART-1^a and ART-1^b) T cell alloantigens to directly identify donor- and host-origin thymocytes in histocompatible adoptive hosts.

In the present study, we adapted these procedures to isolate functional prothymocytes on the FACS. The results indicate that two subsets of prothymocytes are represented in rat bone marrow and spleen, as distinguished by their relative sensitivities to dexamethasone. The prothymocytes in untreated bone marrow have been enriched approximately 110-fold; and the prothymocytes in dexamethasone-treated bone marrow have been enriched approximately 260-fold.

Materials and Methods

Animals. Rats of the LEW, NBR, BUF, and M520 inbred strains were obtained from the Mammalian Genetics and Animal Production Section of the National Cancer Institute, Frederick, MD. Rats used in these experiments were 4–6 wk old, housed two to three per cage, and fed commercial rat chow and acidified, chlorinated water (pH 2.2; free chlorine, 5–10 ppm) ad lib.

Antisera. Antisera to the A.R.T.-1^a and A.R.T.-1^b alloantigens were produced, as previously described, by appropriate reciprocal immunizations and absorptions so as to be functionally specific by indirect immunofluorescence. In some experiments, a monoclonal antibody to the A.R.T.-$1g^a$ alloantigen (termed BC84A) was used and was the kind gift of Dr. John Ely, University of Chicago, Chicago, IL. An $F(ab')_2$ fragment of rabbit IgG anti-rat Thy-1.1 was prepared, and its specificity was determined as before (14, 17).

Immunofluorescence. Single-cell suspensions were prepared in RPMI 1640 tissue culture medium as previously described. Bone marrow and spleen cell suspensions were routinely depleted of erythrocytes by treatment with 0.168 M NH_4Cl before staining for immunofluoresence (15). A.R.T.-1- and Thy-1-labeled cell suspensions were developed for indirect surface immunofluorescence by incubation with an $F(ab')_2$ fragment of an IgG fraction of fluorescein-conjugated rabbit anti-rat or goat anti-rabbit IgG, respectively (N. L. Cappel Laboratories, Cochranville, PA).

Double immunofluorescence for Thy-1 and TdT was performed as follows: bone marrow cells were incubated with monoclonal OX7 antibodies to Thy-1.1 (Accurate Chemical and Scientific Corporation, Westbury, NY) and developed for surface immunofluorescence with rhodamine-conjugated goat anti-mouse IgG that had been passed over a normal rat serum Sepharose 4B affinity column to remove cross-reacting antibodies. The stained cell suspensions were analyzed and sorted on the FACS, smeared onto glass slides using a cytocentrifuge, and fixed in cold absolute methanol for 20 min. The cell smears were then reacted with an affinity-purified IgG fraction of rabbit anti-TdT (18, 19) that was the kind gift of Dr. F. J. Bollum, Uniformed Services, University of the Health Services, Bethesda, MD. The smears were developed for intranuclear immunofluoresence with a fluorescein-conjugated goat anti-rabbit IgG. Doubly labeled cells were counted using a Zeiss Universal Microscope (Carl Zeiss Inc., New York) equipped with narrow band filters for rhodamine and fluorescein.

Cell Sorting. Bone marrow cells were analyzed and sorted on a fluorescence-activated cell sorter (FACS IV, B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) according to low angle light scatter and relative fluorescence intensity for Thy-1.1, as shown in Figs. 1 and 2 (15, 16). Sorted cells were collected in RPMI medium supplemented with 10% gamma globulin-free horse serum.

Irradiation. Recipients received 750 rad total body irradiation from a ^{137}Cs source (Gamma Cell 40 Irradiator, Atomic Energy of Canada, Ltd., Ottowa, Canada; ~120 rad/min) 2–6 h before receiving bone marrow cells.

In Vivo CFU-S. The in vivo spleen colony-forming activity of rat bone marrow and spleen cells was determined by injection of 1×10^6 nucleated cells into irradiated (750 rad) syngeneic

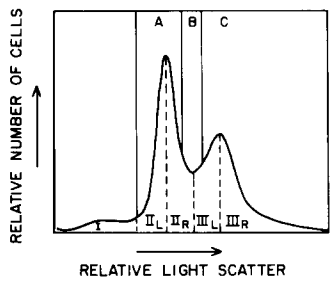

FIG. 1. Relative size distribution of nucleated rat bone marrow cells as determined by low-angle light scatter on the FACS. The various fractions tested for prothymocyte activity are designated by Roman numerals and capital letters. Erythrocytes, which normally present as peak I to the left of the nucleated cells, have been lysed with 0.168 M NH_4Cl.

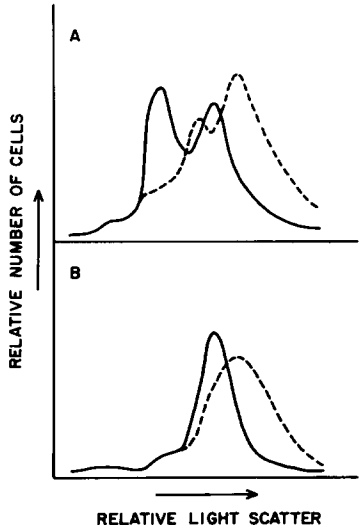

FIG. 2. Relative size distribution of the upper 10th percentile of Thy-1-positive bone marrow cells (-----) superimposed on relative size distribution of total nucleated bone marrow cells (—) from untreated (A) and dexamethasone-treated (B) rats, as determined by low angle light scatter on the FACS.

4–5 wk-old recipients. Spleen colonies were enumerated 11 d later (15).

Thymocyte Regeneration Assay. The quantitative thymocyte regeneration assay using histocompatible, A.R.T.-1-disparate rat strain combinations has been previously described in detail, and was used throughout these studies. Donor-recipient strain combinations for individual experiments are reported in the text. Aliquots of thymocytes in the recipient were routinely stained with the A.R.T.-1^a and A.R.T.-1^b antisera, permitting >95% of the thymocytes to be identified as being of host or donor origin. In each individual experiment, a linear dose-response curve was generated to eliminate interexperimental variations in calculating the relative contents of prothymocytes in the cell populations separated on the FACS. The observed enrichments were determined by comparing the number of fractionated and unfractionated bone marrow cells required to produce an equivalent degree of thymocyte regeneration as determined from the linear dose-response curve. To assure such linear responses, the number of fractionated bone marrow cells injected was adjusted in each instance to approximate the prothymocyte activity contained in 5–15 × 10^6 unfractionated bone marrow cells.

In bone marrow from normal rats, the predicted enrichment of prothymocytes was calculated as the reciprocal of the percentage of total nucleated bone marrow cells present in each fraction.

In bone marrow from dexamethasone-treated rats, the predicted enrichment of prothymocytes was similarly calculated and corrected for the depletion of dexamethasone-sensitive cells in the starting (unfractionated) cell suspension (40% depletion = 1.6-fold enrichment; see Table II).

Dexamethasone Treatment. Donor rats were injected interperitoneally with 0.5 mg dexamethasone per 100 g body weight 72 h before bone marrow or spleen cells were harvested.

Opsonization of Bone Marrow and Spleen Cells. Donor cells were sensitized with anti-Thy-1 antibodies, as described previously (14, 15). Briefly, 1×10^6 nucleated cells were incubated for 20 min at 4°C with 10 μl monoclonal OX7 anti-Thy-1 antibodies (IgG; 1:400 dilution). The cells were washed twice, checked for viability by trypan blue exclusion, and diluted for injection. The presence of mouse immunoglobulin on the treated rat cells was verified by indirect immunofluorescence.

Results

Sensitivity of Prothymocytes to Dexamethasone. The thymocyte regenerating capacities of 50×10^6 normal and dexamethasone-treated M520 strain bone marrow and spleen cells were determined 15 and 16 d, respectively, after injection into irradiated (750 rad) BUF recipients. There was no significant difference between the percentage or absolute numbers of donor-origin (ART-1^a) thymocytes that were generated by bone marrow cells from untreated or dexamethasone-treated rats (Table I). However, 50×10^6 untreated spleen cells generated significantly greater percentages and numbers of donor-origin thymocytes than did 50×10^6 dexamethasone-treated spleen cells.

The thymocyte regenerative capacity of normal vs. dexamethasone-treated bone marrow cells was equivalent at all time points studied up to 140 d after transfer (Fig. 3, $P > 0.8$). In both cases, the percentage of donor-origin thymocytes increased linearly between days 14 and 22 ($r^2 = 0.973$), as previously reported.[1] A high correlation coefficient was observed ($r = 0.986$) when these linear increases in the percentages of donor-origin thymocytes were compared. The long-term stability of the thymic chimeras induced by injection of normal or dexamethasone-treated bone marrow was also equivalent (81.5% vs. 81.2%). Thus, on a per cell basis, the thymocyte progenitor

TABLE I

*Effect of Dexamethasone on Prothymocytes in Rat Bone Marrow and Spleen**

Cell source	Dexamethasone treatment‡	Donor-origin thymocytes (ART-1^a)		Host-origin thymocytes (ART-1^b)	
		Percentage	Number/thymus§ ($\times 10^{-6}$)	Percentage	Number/thymus§ ($\times 10^{-6}$)
Bone marrow	No	23.6	52 ± 16	75.5	168 ± 51
	Yes	28.7	50 ± 11	70.9	124 ± 28
Spleen	No	9.7 ± 4.0	22.9 ± 9.8	89.0 ± 3.8	221 ± 80
	Yes	3.9 ± 2.2‖	9.1 ± 7.2‖	95.5 ± 2.2‖	210 ± 73

* BUF recipients (4 to 6/group), irradiated with 750 rad (bone marrow recipients) or 600 rad (spleen cell recipients), were analyzed on the FACS for the presence of donor- and host-origin thymocytes 15 d after transfer of 50×10^6 M520 bone marrow cells and 16 d after transfer of 50×10^6 M520 spleen cells.

‡ Donor rats were injected intraperitoneally with 0.5 mg dexamethasone 72 h before harvest of bone marrow or spleen cells.

§ Mean ± SD.

‖ Significantly different from untreated controls ($P < 0.001$).

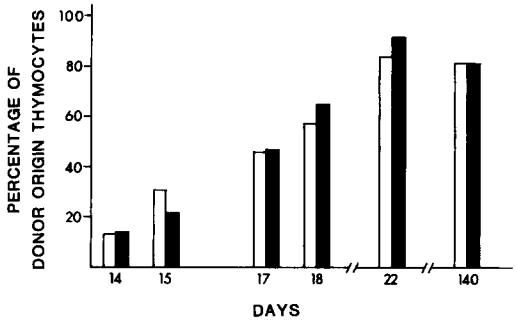

FIG. 3. Generative kinetics of donor-origin thymocytes in irradiated (750 rad) BUF recipients (3–4 per group) injected with 50×10^6 normal (open bars) or dexamethasone-treated (closed bars) M520 bone marrow cells, as determined by analysis on the FACS. A linear increase with time is seen between days 14 and 22 in the percentage of donor-origin thymocytes in recipients of both normal ($r^2 = 0.974$) and dexamethasone-treated ($r^2 = 0.973$) bone marrow (correlation coefficient $r = 0.986$). There were no significant differences ($P > 0.8$) observed at any point over a 140-d period between the thymocyte generative capacities of equal numbers of normal or dexamethasone-treated bone marrow cells.

TABLE II

*Effects of Dexamethasone on Rat Bone Marrow and Spleen Cells**

Cell source	Dexamethasone treatment	Nucleated cells per femur or spleen ($\times 10^{-6}$)	Thy-1-positive cells‡	TdT-positive cells‡	CFU-S/10^6 cells§
			%	%	
Bone marrow	No	156 ± 21	36.8 ± 3.5	5.7 ± 0.7	23 ± 5
	Yes	98 ± 8‖	14.1 ± 3.7‖	0.9 ± 0.3‖	44 ± 8‖
Spleen	No	374 ± 30	12.5 ± 1.1	<0.1	8 ± 1
	Yes	144 ± 19‖	6.6 ± 1.4‖	<0.1	21 ± 3‖

* Analysis of 4–6 spleens and femurs from untreated or dexamethasone-treated M520 rats (mean ± SD).
‡ Determined by indirect immunofluorescence.
§ Spleen colonies were enumerated 11 d after injection of 1×10^6 nucleated bone marrow or spleen cells into syngeneic recipients irradiated with 750 rad.
‖ Significantly different from untreated controls ($P < 0.001$).

activity in normal and dexamethasone-treated rat bone marrow was the same.

These results contrasted with the effects of dexamethasone on other cell types in bone marrow and spleen. As shown in Table II, dexamethasone treatment caused a significant decrease in the total numbers of nucleated cells in bone marrow (40%, $P < 0.001$) and spleen (65%, $P < 0.001$). As reported previously (15), the decrease in bone marrow cells was almost entirely accounted for by a decrease in Thy-1-positive lymphoid cells, including a marked decrease in TdT-positive cells. The decrease in spleen cells was due primarily to a decrease in Thy-1-negative lymphoid cells. Conversely, CFU-S, which are cortisone-resistant (15), were proportionately enriched in bone marrow and spleen after treatment with dexamethasone. Had all prothymocytes been dexamethasone-resistant, comparable enrichments should have been observed. The fact that no enrichment of prothymocytes occurred in bone marrow after dexamethasone treatment, and a decrease occurred in spleen, suggests that ~40% of prothymocytes in bone marrow and ~80% of prothymocytes in spleen are dexamethasone sensitive.

Light Scatter Profile of Prothymocytes. Normal rat bone marrow cells were separated into various fractions according to the low angle light scatter profile (relative size distribution) observed on the FACS (Fig. 1). As previously described, peak I consists of erythrocytes, peak II contains mainly lymphocytes and some erythroblasts, and peak III contains undifferentiated blast cells and cells of the myeloid series (15). Most CFU-S activity is present in the left side of peak III (fraction III_L), and most TdT-positive cells are present in the valley between peaks II and III (fraction B) (15, 16).

Results in Table III show the relative size distribution profile of presumptive prothymocytes. No prothymocyte activity was detectable in peak II; ~25% of total prothymocyte activity was present in fraction B; and ~75% of the prothymocyte activity was present in fraction C. Further analysis showed that ~75% of the total thymocyte regenerating activity was present in fraction III_L. Therefore, by process of exclusion, ~50% of the total prothymocyte activity was located in that portion of fraction III_L that was to the right of fraction B; and 25% of the prothymocyte activity was in fraction III_R.

Opsonization of Prothymocytes with Anti-Thy-1 Antibodies. Results in Table IV show that all thymocyte regenerating activity was abrogated by treating M520 donor strain rat spleen or bone marrow cells with monoclonal IgG anti-Thy-1.1 antibodies immediately before injection into irradiated BUF strain recipients. As anticipated (15), CFU-S activity was also eliminated by this procedure. These results suggested that prothymocytes, like CFU-S and other immature hemopoietic cell types in the rat, are Thy-1 positive. Direct evidence for this contention was provided by FACS analysis (see below).

Fluorescence Distribution Profile of Prothymocytes. The thymocyte regenerating activity of rat bone marrow cells that had been treated with an $F(ab')_2$ fragment of rabbit anti-rat-Thy-1.1 and developed for immunofluorescence with an $F(ab')_2$ fragment of fluorescein-conjugated goat anti-rabbit IgG was only slightly less than that of unlabeled bone marrow cells (Table V, line 10). Moreover, passage of bone marrow cells through the FACS did not significantly affect their thymocyte regenerative potential. Hence, it was possible to sort rat bone marrow cells on the FACS according to their

TABLE III

Distribution of Prothymocytes in Rat Bone Marrow According to Relative Low Angle Light Scatter

Fraction*	Percentage of total nucleated bone marrow cells per fraction	Observed enrichment of prothymocytes‡	Expected enrichment of prothymocytes
B	20.1	1.0	5.0
C	39.5	2.3	2.5
II	42.3	<1.0	2.4
III	57.7	1.6	1.7
III_L	25.9	4.7	3.9
III_R	26.2	1.0	3.8

* Bone marrow cells were fractionated on the FACS, as described in Fig. 1.

‡ Calculated as described in Materials and Methods. Donor-recipient combinations included M520 into BUF and NBR into LEW strain rats. The percentages of donor- and host-origin thymocytes were determined 16–20 d after transfer of bone marrow cells. Results represent means of 2–4 experiments.

TABLE IV

*Effect of Anti-Thy-1 Serum on Prothymocytes and CFU-S in Rat Bone Marrow and Spleen**

Cell source	Treatment	CFU-S/10^6 cells	Donor-origin thymocytes		Host-origin thymocytes	
			Percentage	Number/ thymus ($\times 10^{-6}$)	Percentage	Number/ thymus ($\times 10^{-6}$)
Bone Marrow	Medium	22.4 ± 6.0	42.1 ± 13.7	70.7 ± 36.4	56.0 ± 14.0	94.6 ± 41.6
	Anti-Thy-1.1	0	<1.0	<1.0	96.8 ± 0.4	67.0 ± 76.0
Spleen	Medium	11.5 ± 2.1	10.4	13.7	82.2	113
	Anti-Thy-1.1	0	<1.0	<1.0	95.3	137

* Anti-Thy-1.1 (OX7) or medium-treated M520 spleen and bone marrow cells were injected into 750 rad-irradiated BUF recipients (3–4/group). Spleen colonies were counted 11 d after injection of 10^6 nucleated cells. The percentage of donor- and host-origin thymocytes in aliquots of thymus cells from individual bone marrow recipients or pools of thymocytes from spleen cell recipients was determined by fluorescence analysis on the FACS 16 d after transfer of 50×10^6 nucleated cells. Results are expressed as mean ± SD.

TABLE V

Distribution of Prothymocytes in Rat Bone Marrow According to Relative Fluorescence Intensity for Thy-1 Antigen

Thy-1-positive cells (percentile)*	Untreated bone marrow cells			Dexamethasone-treated bone marrow cells		
	Percentage of total nucleated bone marrow cells per fraction	Observed enrichment of prothymocytes‖	Predicted enrichment of prothymocytes‖	Percentage of total nucleated bone marrow cells per fraction	Observed enrichment of prothymocytes‖	Predicted enrichment of prothymocytes‖
91–100 (fraction III_L)‡	1.1	112	90	0.35 (0.22)¶	260	286 (458)¶
91–100	4.5	21	22	2.1 (1.3)	45	48 (77)
1–90	40.8	<1	2.5	21.0 (13.2)	<1	4.8 (7.7)
Thy-1⁻ plus 1–90	96.5	<1	1	97.7 (61.3)	<1	1.0 (1.6)
76–100	11.1	6.3	9	ND**	ND	ND
76–90	ND	ND	ND	3.3 (2.1)	<1	30 (48)
1–75	33.2	<1	3	16.6 (10.4)	<1	6 (9.6)
Thy-1⁻ plus 1–75	87.5	<1	1.1	93.2 (58.4)	<1	1.1 (1.8)
Thy-1-negative	56.2	<1	1.8	78.0 (48.3)	<1	1.3 (2.1)
Unseparated§	100.0	1	1	100.0 (62.7)	0.9	1.0 (1.6)

* Percentiles of Thy-1-positive cells were separated on the FACS according to relative fluorescence intensity (see ref. 16 for details). Results represent means of 2–4 experiments.

‡ Upper 10th percentile of Thy-1-positive cells in fractions III_L (Figs. 1 and 2).

§ Comparable results were obtained with unlabeled, unsorted bone marrow cells and with anti-Thy-1-labeled, FACS sorted (but unseparated) bone marrow cells.

‖ Calculated as described in Materials and Methods. Values of <1 indicate that no prothymocytes were detected.

¶ Values in parentheses are corrected for depletion of dexamethasone-sensitive cells in starting (unfractionated) cell suspension.

** Not determined.

relative fluorescence intensity for the Thy-1.1 antigen and to assess the thymocyte regenerating capacities of the various cell fractions.

Results in Table V show that all prothymocyte activity in normal rat bone marrow resides in the upper 10th percentile of Thy-1-positive cells, as judged by relative fluorescence intensity. Separation of this fraction, which represented ~4.5% of total nucleated bone marrow cells, was accompanied by a 21-fold enrichment of prothymocyte activity, as predicted. No prothymocyte activity was found in the Thy-1-

negative cell fraction or in the lower 90th percentile of Thy-1-positive cells.

Combining the results obtained from light scatter analysis (Table III) with results based on Thy-1 fluorescence intensity (Table V), the predicted enrichment of prothymocytes in the upper 10th percentile of Thy-1-positive cells in fraction III_L (Figs. 1 and 2), representing 1.1% of total nucleated bone marrow cells, was approximately 90-fold. The observed enrichment in this cell fraction was 112-fold (Table V, line 1).

Much greater enrichment of prothymocyte activity was obtained when bone marrow cells from dexamethasone-treated donors were sorted according to the relative intensity of Thy-1 fluorescence (Table V). Separation of the upper 10th percentile of total Thy-1-positive cells, representing ~2.1% of dexamethasone-resistant bone marrow cells, resulted in a 45-fold enrichment of prothymocyte activity; and separation of the upper 10th percentile of Thy-1-positive cells in fraction III_L (Figs. 1 and 2), representing 0.35% of total dexamethasone-resistant cells, resulted in a 260-fold enrichment of prothymocyte activity. Again, the observed enrichments were ~40% lower than what would have been predicted had all prothymocytes been dexamethasone-resistant. Hence, injection of as few as 2.9×10^4 cells from the prothymocyte-enriched cell fractions of dexamethasone-treated bone marrow donors generated 7.3% (6.6×10^6) donor-origin thymocytes by 16 d after cell transfer, whereas 7.5×10^6 unfractionated bone marrow cells were required to generate equivalent numbers of donor-origin thymocytes.

Fluorescence Distribution Profile of TdT-positive Cells. The distribution of TdT-positive cells in bone marrow from untreated and dexamethasone-treated rats was determined on the FACS according to relative fluorescence intensity for Thy-1 antigen. The results in Table VI are comparable to those published previously (16). Thus, ~75% of total TdT-positive cells in untreated bone marrow were present in the upper 25th percentile of Thy-1-positive cells, and >80% of TdT-positive cells were sensitive to dexamethasone.

Comparison with results in Tables V and VI shows that, although both TdT-positive cells and prothymocytes are each strongly Thy-1 positive, there are major disparities in both their distribution profiles and degrees of enrichment. Thus, in both untreated and dexamethasone-treated bone marrow, ~70% of total TdT-positive cells were present in the lower 90th percentile of Thy-1-positive cells in which no prothymocyte activity is detectable. Moreover, in the fraction in which both TdT-positive

TABLE VI

*Distribution of TdT-Positive Cells in Rat Bone Marrow According to Relative Fluorescence Intensity for Thy-1 Antigen**

Thy-1-positive cells (percentile)	Untreated bone marrow cells			Dexamethasone-treated bone marrow cells		
	Percentage of total TdT-positive cells per fraction	Percent TdT-positive cells per fraction	Observed enrichment of TdT-positive cells	Percentage of total TdT-positive cells per fraction	Percent TdT-positive cells per fraction	Observed enrichment of TdT-positive cells
91–100	30.1 ± 8.1	17.5 ± 4.3	3.1	28.4 ± 3.6	13.5 ± 4.5	15.0
76–90	43.3 ± 5.5	17.9 ± 5.1	3.1	42.4 ± 12.4	13.1 ± 6.4	14.6
1–75	26.6 ± 10.6	2.1 ± 1.3	0.4	29.2 ± 12.5	1.6 ± 0.7	1.8
Thy-1-negative	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1
Unseparated	100	5.7 ± 0.7	1.0	100	0.9 ± 0.3	1.0

* Percentiles of Thy-1-positive cells were separated on the FACS according to relative fluorescence intensity (surface rhodamine staining), and methanol-fixed smears of the separated cell fractions were stained with anti-TdT antibodies (intranuclear fluorescein staining) (see Materials and Methods). Results represent the means ± SD of three experiments.

cells and prothymocytes are present (upper 10th percentile of Thy-1-positive cells), the enrichment of prothymocytes was much greater than that of TdT-positive cells (21-fold vs. 3-fold in untreated bone marrow; 45-fold vs. 15-fold in dexamethasone-treated bone marrow).

Discussion

The results of this study point to the existence of two subsets of prothymocytes, one of which is sensitive to dexamethasone, the other of which is not. Dexamethasone treatment neither enriched nor depleted bone marrow prothymocyte activity, suggesting that the proportional distribution of these two prothymocyte subsets roughly approximates that of other dexamethasone-sensitive and -resistant bone marrow cells. Thus, it can be calculated that ~40% of prothymocytes in bone marrow and ~80% of prothymocytes in spleen are dexamethasone-sensitive. This interpretation is strongly supported by a proportionate enrichment of CFU-S in bone marrow and spleen of dexamethasone-treated donors. Unlike prothymocytes, essentially all CFU-S are resistant to adrenal glucocorticosteroids (15). Our results are also consistent with those of Basch and Kadish (20), who observed only a minimum enrichment of prothymocyte activity in bone marrow and spleen from hydrocortisone-treated mice. An alternative explanation for the present results is the depletion of a dexamethasone-sensitive population of regulatory cells that influence the function of dexamethasone-resistant prothymocytes. However, recent mixing experiments using untreated and dexamethasone-treated bone marrow cells have not provided a basis for the existence of such regulatory cells (unpublished observations).

Aside from their differential sensitivities to dexamethasone, we did not detect other distinguishing features between the two subsets of presumptive prothymocytes. Both were similar with respect to relative light scatter, relative fluorescence intensity for Thy-1 antigen, and relative thymocyte regenerating capacity. Given that prothymocyte activity was assayed 72 h after administration of dexamethasone, it is possible that dexamethasone-sensitive prothymocytes were generated during this period by dexamethasone-resistant precursors.

The presence of high concentrations of Thy-1 antigen on functional prothymocytes extends the known distributions of this marker on immature lymphohemopoietic cells in the rat. Previous studies (11–16, 21, 22) demonstrated Thy 1 antigen on pluripotent hemopoietic stem cells, myeloid (and probably erythroid) progenitor cells, TdT-positive bone marrow cells, cortical thymocytes, and immature members of the B lymphocyte lineage.

In the rat, bone marrow precursors of peripheral T cells have been enriched approximately four- and eight-fold by sorting on the FACS according to relative fluorescence intensity for Thy-1 and W3/13 antigens, respectively (23, 24). Although the identity of these precursors is unknown, it is likely that they are actively proliferating cells (25). However, there is no direct evidence that these T lymphocyte regenerating cells are prothymocytes.

Although the quantitative thymocyte regeneration assay permits accurate comparisons to be made of the relative prothymocyte contents of various cell suspensions, it does not allow the absolute numbers of prothymocytes to be directly quantified.[1] This is due to the long lag period (10–12 d) that precedes the onset of the linear growth phase of donor-origin thymocytes and the absence of information concerning the

seeding efficiency of prothymocytes to the thymus of the adoptive host. Nonetheless, an estimate of the maximum numbers of dexamethasone-resistant prothymocytes in the 260-fold enriched fraction can be made. Thus, using a similar protocol to isolate CFU-S on the FACS (15), we determined that at least 40% of the cells in the upper 10th percentile of Thy-1-positive cells in fractions II_R and III_L (mostly III_L) from cortisone-treated donor rats are CFU-S. Myeloid and erythroid progenitors are not significantly represented in this fraction; and ~20% of the cells are basophilic erythroblasts. Therefore, if it is assumed that the remaining 40% of the cells in this fraction are prothymocytes, it can be calculated that a maximum of 0.15% of total dexamethasone-resistant bone marrow cells are prothymocytes (40% × 0.35%; see Table V, line 1). A similar calculation suggests that a maximum of 0.25% of cells in untreated bone marrow are prothymocytes. This value is comparable to that assigned to myeloid and erythroid progenitor cells (26).

In contrast to the above estimates of the frequency of functional prothymocytes in rat bone marrow, it has been observed (9, 27, 28) that between 5 and 10% of cells in bone marrow and spleen from both normal and congenitally athymic mice can be induced by a variety of factors to express T cell-specific differentiation antigens. Some of these cells have the ability to migrate to the thymus of irradiated recipients (29). It has, therefore, been presumed that many, perhaps most, of these inducible cells are prothymocytes. If it is assumed for the purpose of argument that 5% of bone marrow cells are prothymocytes, then a maximum enrichment of only 20-fold is possible. Yet, we attained prothymocyte enrichments of 112-fold for untreated bone marrow and 260-fold for dexamethasone-treated bone marrow, with recoveries of ~75% in each instance. Three likely explanations for this discrepancy are evident: (*a*) most inducible prethymic cells do not function as prothymocytes (i.e., they can not migrate to or significantly proliferate in the thymus); (*b*) the adoptive transfer system only detects a minor subset of prothymocytes; (*c*) the thymocyte generative activity in our assay is due to pluripotent hemopoietic stem cells (CFU-S). There is no reason to believe that the difference between the percentage of inducible prethymic cells in the mouse and of functional prothymocytes in the rat reflects major differences in the representation of prothymocytes in these species. Roughly equivalent numbers of bone marrow and spleen cells, when normalized for body weight, are required to regenerate thymocytes in mice and rats; and the kinetics of regeneration and the threshold of irradiation are identical in both species (5, 30).[1] Moreover, above a critical dose of irradiation (~400 rad), the thymocyte regeneration assays in both mice and rats are strictly dose-dependent. Therefore, it is unlikely that the small number of detectable prothymocytes in rat bone marrow is a unique consequence of our assay system.

Although additional experiments will be required to resolve the preceding issues, it is tempting to speculate on the identity of the prothymocytes that we have enriched on the FACS. It seems safe to conclude that, at a minimum, the dexamethasone-sensitive prothymocytes are not CFU-S, almost all of which are cortisone-resistant (15). *A priori* reasoning would suggest, instead, that the dexamethasone-sensitive prothymocyte subset in bone marrow is composed of TdT-positive cells. Thus, in the mouse, the majority of TdT-positive bone marrow cells can be induced to express T cell differentiation antigens, and the majority of inducible bone marow cells are TdT-positive (9). Moreover, most cortical thymocytes are also TdT-positive, but medullary thymocytes and peripheral T cells are TdT negative (31, 32). However, there was no

correlation in the present study between the enrichment of TdT-positive bone marrow cells and the enrichment of prothymocytes. Indeed, 75% of the total TdT-positive cells were excluded from the prothymocyte-rich fraction. Furthermore, a population of dexamethasone-sensitive prothymocytes was also identified in adult rat spleen, which lacks TdT-positive cells. None of this excludes the possibility that TdT-positive bone marrow cells may function as prothymocytes in the intact host. It is equally possible that, for technical reasons, TdT-positive bone marrow cells cannot function as prothymocytes in our adoptive transfer assay system or that only a minor subset of TdT-positive cells can function as prothymocytes at any particular time. Moreover, although TdT-positive cells are not normally present in adult spleen, it should be noted that many prethymic cells in spleen can be rapidly induced to express TdT (9).

The identity of the dexamethasone-resistant prothymocyte subset is also problematic, especially because it coisolates with CFU-S on the FACS (15). However, there is considerable evidence from other studies that functional prothymocytes are distinct from CFU-S, and that they constitute a developmentally and physically discrete population of actively proliferating cells in hemopoietic tissues (6–8, 25, 33).

In studies in progress, we demonstrated that the dexamethasone-resistant prothymocytes can generate TdT-positive thymocytes in vivo, thereby suggesting that they belong to the committed subset of pre-TdT-positive cells (9). In addition, we recently used a selective culture system (34) to generate TdT-positive rat bone marrow cells in vitro. We found that the bone marrow cell fraction that is enriched for dexamethasone-resistant prothymocytes is comparably enriched for cells that can generate undifferentiated TdT-positive cells in vitro (unpublished observations). The growth kinetics of these TdT-positive cells suggest that they are derived from actively proliferating precursors, which, therefore, are unlikely to be CFU-S. Our working hypothesis is that the in vivo precursors of thymocytes and the in vitro precursors of TdT-positive cells belong to a common pool of dexamethasone-resistant prothymocytes. We are presently attempting to further characterize this highly enriched population of prothymocytes and to physically separate it from CFU-S. We are also attempting to determine whether TdT-positive cells and other undifferentiated cells that can be induced to express T cell markers can function as prothymocytes in other thymocyte regeneration assays. For the moment, however, the objective evidence is that functional prothymocytes constitute a much smaller subset of bone marrow cells than had heretofore been suspected.

Summary

A quantitative thymocyte regeneration assay was used to monitor the isolation of functional prothymocytes from rat bone marrow on the FACS. Two prothymocyte subpopulations were tentatively identified on the basis of their relative resistance to dexamethasone. Both populations were comprised of undifferentiated, medium-size cells that displayed large amounts of Thy-1 antigen. Simultaneous sorting of bone marrow cells according to relative low angle light scatter (size) and relative fluorescence intensity for Thy-1 resulted in enrichments of 112-fold and 260-fold, respectively, in prothymocyte activity in untreated and dexamethasone-treated bone marrow. These prothymocyte-enriched cell fractions contained ~75% of total functional prothymocyte activity in bone marrow, and represented 1.1 and 0.35% of total untreated and dexamethasone-treated bone marrow cells. Using these enriched cell fractions,

significant thymocyte regeneration is possible with as few as 2×10^4 and 1×10^4 bone marrow cells, respectively. The possible relationship of these functional prothymocyte subpopulations with CFU-S and with TdT-positive cells is discussed.

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