TWO RARE POPULATIONS OF MOUSE Thy-1° BONE MARROW CELLS REPOPULATE THE THYMUS

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The ontogeny of mammalian cell-mediated immune responsiveness is critically dependent upon the specialized maturational microenvironment of the thymus. The initial event heralding the lymphoid development of the thymus occurs (in the mouse) at approximately the eleventh day of gestation, when lymphoid stem cells, apparently derived from the yolk sac or fetal liver, migrate into the largely epithelial thymic anlage (1, 2). These lymphoid stem cells establish an intrinsic thymic pool of precursor cells with limited self-renewing potential; the lymphoid cells of the thymus are maintained throughout adult life due to a continual low-level seeding of the thymus by precursor cells of extrathymic (mostly bone marrow) origin (3). Much attention has been devoted to characterizing the bone marrow–derived T cell precursors, since at least some congenital abnormalities in cell-mediated immune function are thought to result from defects within this population of bone marrow cells (4). Further, isolation of bone marrow–derived T cell precursors may prove to be clinically important in repopulating the thymic lineages of cells after viral-, radiation-, or chemically induced immunodeficiencies.

We have previously identified and isolated a small fraction (0.1–0.3%) of mouse bone marrow cells that contains pluripotent hematopoietic stem cells (5). This population of stem cells expresses low levels of the Thy-1 antigen (Thy-1°) and lacks expression of surface markers that characterize cells within defined differentiated hematolymphoid cell lineages (Thy-1° Lin−). Elsewhere, we have shown that this population contains B cell precursors and myeloid/erythroid precursor activity (5). The isolated fraction of cells was able to rescue lethally irradiated mice and repopulate the thymus and peripheral T cell populations (6). This experiment implies, but does not prove, that the putative stem cell fraction is able to home to the thymus and repopulate the T cell lineage. Therefore, we sought to quantitate T cell precursor activity within the bone marrow by using a quantitative limiting dilution assay of thymic homing. We report here that the Thy-1° Lin− bone marrow fraction contains thymus-homing T cell precursors at...
a level that is 50–80-fold enriched over unseparated bone marrow cells. A second fraction of mouse bone marrow, which is also Thy-1° but expresses at least one other hematolymphoid lineage marker (Thy-1° Lin+), is present as 1–5% of the nucleated cells and contains a twofold enrichment of thymic repopulating activity. These two fractions can account for at least half of the thymic repopulating activity in the bone marrow.

Materials and Methods

Mice. C57BL/Ka (Thy-1.2, Ly1.2, Ly5.1), C57BL/6-α17-Ly1.1 (B/6-Ly1.1; Thy-1.2, Ly1.1, Ly5.1), and C57BL/Ka-Thy-1.1 (BA; Thy-1.1, Ly1.2, Ly5.1) were bred and maintained in our animal facility. C57BL/6J-Ly5.2: Pep-3° (B/6-Ly5.2; Thy-1.2, Ly1.2, Ly5.2) breeding pairs were kindly provided by Dr. E. A. Boyse (Memorial Sloan Kettering Cancer Center, NY, NY) and thereafter were bred and maintained in our animal facility.

Antibodies. Mouse mAbs 19XE5 (anti-Thy-1.1), obtained from Dr. R. Nowinski (Genetic System Inc., Seattle, WA), and A20.1 (anti-Ly5.2) and 104.2 (anti-Ly5.1), obtained from Dr. E. A. Boyse, were purified by affinity chromatography on immobilized protein A and were derivatized with fluorescein or biotin by standard techniques. Mouse mAb MAR-18 (anti-rat κ light chain) and rat mAbs 53-2.1 (anti-Thy-1.2), M1/70.15.11.5 (anti-Mac-1), GK1.5 (anti-CD4), and 55-6.72 (anti-CD-8) were obtained from the American Type Culture Collection, Rockville, MD. Rat mAbs RB6-8C5 (anti-Gr-1, a granulocyte marker) and RA3-6B2 (anti-B220), were obtained from Dr. R. Coffman (DNAX, Stanford, CA) (7,8). Rat mAbs were concentrated for fluorescein or biotin conjugation by ammonium sulfate precipitation or membrane filter concentration of serum-free culture supernatants.

Multiparameter Cell Sorting. The dual laser FACS used in these experiments was modified from a Becton Dickinson & Co. (Mountain View, CA) machine as described (9). This instrument and supporting computer hardware and software were made available through the FACS-shared users group at Stanford University, Stanford, CA.

Preparation of Bone Marrow Suspensions. Fresh bone marrow cells were isolated by flushing femora from 3-wk-old mice with HBSS containing 5% FCS and 10mM Hepes buffer Suspensions to be separated by FACS were reacted with fluoresceinated anti-Thy-1.1 or anti-Thy-1.2 antibodies together with a cocktail of biotinylated rat mAbs specific for mouse differentiation antigens Gr-1, Mac-1, B220, CD4, and CD8 (see above for antibody designations). After a wash in HBSS through a cushion of FCS, the cells were reacted with Texas red–coupled avidin (Cappel Laboratories, Cochranville, PA). After a second wash, the cells were resuspended for FACS separation. Debris, erythrocytes, doublets, and dead cells were excluded by forward scatter and propidium iodide gating.

Thymic Repopulation Assay. The limiting dilution method of Ezine et al. (10) was used to detect thymic-homing T cell precursors. Briefly, normal 4–8-wk-old male or female C57BL6/Ka mice were irradiated (700 rad at 100 rad/min from a Phillips 250 Kv x-ray machine). The animals were reconstituted 3–24 h later with an intravenous transfer of 2.5 × 10⁶ bone marrow cells of syngeneic origin. In addition, unseparated or FACS-isolated fractions of bone marrow from congenic mice were included in the intravenous transfer. The transferred bone marrow cells differentiate in vivo to give rise to populations of thymocytes and mature T cells, within which can be detected the progeny of the congenic population by mAbs specific for the allelic cell surface marker. If the congenic population of bone marrow cells is present at limiting dilution, only a fraction of the reconstituted animals will contain progeny from that population. Each clonogenic cell gives rise to a colony, or focus, of donor-derived cells in the thymus (10).

The recipient animals were killed 5–6 wk after reconstitution and analyzed by either immunohistochemical techniques or by two-color FACS for the presence of T cells derived from the donor strains, based on the expression of the relevant allele. In all cases, the thymus and peripheral lymph nodes were analyzed. Animals with significant populations (>0.1% of the cells analyzed) of donor-derived cells in thymus or lymph
FIGURE 1. Detection of donor-derived cells by FACS analysis. Thymocytes from two radiation chimeras that received intrathymic injections of Thy-1<sup>+</sup> Lin<sup>-</sup> cells sorted from a Thy-1.1 congenic donor 4 wk earlier were stained for the Thy-1.1 allele. Fluorescent events are scored as positive cells only if they fall within the fluorescent intensity and forward plus right angle scatter windows defined by control positive cells. (A) A typical negative animal in which no positive cells were detected in 50,000 events. (B) The threshold of detection as 15 positive cells in 50,000 events (0.03%). In practice, animals are considered positive when donor-derived cells comprise 0.1% of the organ. Therefore, in a typical thymic lobe containing \( 5 \times 10^7 \) cells, the threshold clone size is \( 5 \times 10^4 \) cells. Logarithmic contour plots have been used to facilitate visualization of the rare population of donor cells (9) FACS analysis of lymph node suspensions is equally sensitive (data not shown).

Intrathymic injections of bone marrow or bone marrow fractions were performed under ether or ketamine/xylazine anesthesia (11). Mice were irradiated (700–800 rad) within 3 h before surgery. The indicated number of cells were injected into one thymic lobe of each recipient mouse in a volume of 5 μl. Animals were killed 3–4 wk after injection and a cell suspension was prepared from each thymic lobe. The suspensions were stained and analyzed as above.

We have proposed the term “thymic colony-forming unit” (CFU)<sup>1</sup> to denote the bone marrow cells that give rise to the thymic colonies (12). This nomenclature leaves open the question of whether the colonies are the products of committed thymocyte progenitors (prothymocytes, pre-T cells) or of pluripotent hematopoietic stem cells. Thymus-homing CFU, denote these progenitors of thymic colonies that appear after intravenous, as opposed to intrathymic, transfer of bone marrow cells.

Results

It is known that the bone marrow contains cells capable of homing to and repopulating the thymus of irradiated animals (13). To characterize the cells within the bone marrow that express this thymus homing CFU, activity, clonogenic thymus-homing bone marrow cells were enumerated as previously described (10) after FACS isolation of defined bone marrow subsets. Fig. 2 shows a representative contour plot obtained by staining a suspension of bone marrow cells for expression of Thy-1 in the fluorescein dimension and for

<sup>1</sup> Abbreviations used in this paper: CFU<sub>s</sub>, splenic CFU; CFU<sub>t</sub>, thymic CFU.
Two Populations of Colony-Forming Units in Bone Marrow

Figure 2. Bone marrow cell suspension staining for isolation of hematopoietic stem cells. Fresh bone marrow cells were isolated from 3-wk-old C57Bl/Ka-Thy-1.1 (BA) mice. Cell staining was performed as described in Materials and Methods. (A) The four fractions of cells (I–IV) contained the following percentages of the total population: I, 76%; II, 20%; III, 3%; IV, 0.3%. Solid lines indicate background levels of staining. (B) The cells contained in fraction IV express roughly 10-fold lower levels of the Thy-1 antigen than do mature T lymphocytes, which are visualized as the isolated population in A, fraction III. B was generated during the collection of Thy-1<sup>+</sup> cells, and as such reflects only the sorting windows, not the purity, of the populations. Upon reanalysis, Thy-1<sup>+</sup> Lin<sup>−</sup> cells collected by cell sorting were at least 60% pure, with the contaminating cells being derived mostly from fraction I. Note that they Thy-1<sup>+</sup> Lin<sup>−</sup> cells excluded mature T lymphocytes, which are the Thy-1<sup>+</sup> Lin<sup>+</sup> population in fraction III.

Do Thy-1<sup>+</sup> Lin<sup>−</sup> bone marrow cells include clonogenic, thymus-homing CFU<sub>H</sub> cells? The data shown in Table I summarize the results of four independent experiments. The cotransfer of various number of congenic bone marrow cells with 2.5 × 10<sup>6</sup> syngeneic unseparated bone marrow cells into irradiated recipient mice results in congenic-derived thymic colonies in some of the animals. The fraction of positive animals is directly dependent upon the number of congenic haplotype cells that are transferred. Application of limiting dilution statistics to the unseparated bone marrow data reveals that one thymus-homing CFU<sub>H</sub> is engrafted when 3.4 × 10<sup>4</sup> congenic bone marrow cells are cotransferred with 2.5 × 10<sup>6</sup> syngeneic bone marrow cells, with a coefficient of correlation r = −0.98. Thymus-homing CFU<sub>H</sub> activity was enormously enriched when FACSS-isolated Thy-1<sup>+</sup> Lin<sup>−</sup> bone marrow cells were transferred (Table I). Individual thymic clones were observed when only 100–250 Thy-1<sup>+</sup> Lin<sup>−</sup> cells from congenic donors were included in the reconstituting population. This enrichment is comparable to the ~100–200-fold increase in pre-B and CFU activities in the Thy-1<sup>+</sup> Lin<sup>−</sup> fraction when compared with unfractionated bone marrow (5).
While the greatest enrichment of detectable thymus-homing CFU, in bone marrow was contained in the Thy-1⁺⁻ Lin⁻ population, some activity was also found in the bone marrow population that coexpresses Thy-1 and at least one of the determinants detected by the cocktail of antibodies specific for other maturational markers (Thy-1⁺⁻ Lin⁺, Table I). The enrichment does not approach that seen in the Thy-1⁺⁻ Lin⁻ fraction when compared with unseparated bone marrow, and is not due to mature bone marrow T cells (Thy-1hi CD4⁺ and/or CD8⁺). Finally, the data in Table I show that the fraction of bone marrow that lacks expression of either Thy-1 or any of the maturation-associated determinants (Thy-1⁻⁻ Lin⁻⁻) also lacks thymus-homing CFU, activity. Thus, the greatest enrichment of bone marrow cells capable of homing to and differentiating within the thymus was contained in the 0.1–0.3% of bone marrow with the phenotype Thy-1⁺⁻ Lin⁻⁻, while an equivalent amount of CFU, activity was found at a 10-fold lower enrichment in the Thy-1⁺⁺⁻, Lin⁺ fraction.

The data in Table I demonstrate a 50–100-fold enrichment of thymus-seeding clonogenic CFU, in the Thy-1⁺⁻ Lin⁻⁻ population. Approximately 1 in 600 of these cells produced a clonal thymic repopulation after intravenous injection, as compared to 1 in 34,000 unseparated bone marrow cells. It is possible that an even higher proportion of the Thy-1⁺⁻ Lin⁻⁻ cells can respond to the thymic microenvironment, since only a fraction of the Thy-1⁺⁻ Lin⁻⁻ cells placed in the bloodstream were likely to seed to the thymus.

To evaluate critically the absolute frequency of CFU, contained within the

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**Table I**

| Cell population          | Number of cells transferred | Positive animals/total | Percent of donor-derived cells mean (range) |
|--------------------------|-----------------------------|------------------------|-------------------------------------------|
| Unseparated bone marrow  | 5.0 × 10⁴                    | 18/24                  | ND*                                       |
|                          | 2.5 × 10⁴                    | 8/14                   | 1.5% (0.1–1.5)                            |
|                          | 1.7 × 10⁴                    | 5/16                   | ND                                        |
|                          | 8.3 × 10³                    | 3/19                   | ND                                        |
| Thy-1⁺⁻, Lin⁻⁻           | 2.5 × 10⁵                    | 6/6                    | ND                                        |
|                          | 1.4 × 10⁵                    | 5/5                    | ND                                        |
|                          | 2.5 × 10⁴                    | 3/9                    | 0.5% (0.1–1.2)                            |
|                          | 1.8 × 10⁵                    | 2/5                    | ND                                        |
|                          | 1.0 × 10⁶                    | 1/3                    | 0.5                                       |
| Thy-1⁺⁺, Lin⁺⁺           | 2.5 × 10⁵                    | 4/4                    | 3.7% (0.2–11.8)                           |
|                          | 1.4 × 10⁵                    | 1/5                    | ND                                        |
|                          | 1.8 × 10⁶                    | 0/5                    |                                            |
| Thy-1⁺⁺ CD4⁺ and/or CD8⁺ | 5.0 × 10⁶                    | 0/3                    |                                            |
| Thy-1⁻⁻, Lin⁻⁻           | 1.0 × 10⁶                    | 0/4                    |                                            |
|                          | 2.5 × 10⁷                    | 0/6                    |                                            |
|                          | 2.5 × 10⁸                    | 0/6                    |                                            |

* The percentage of donor cells was not determined in groups analyzed by immunohistochemical staining.
TWO POPULATIONS OF COLONY-FORMING UNITS IN BONE MARROW

FIGURE 3. Limiting dilution analysis of CFU, activity in whole bone marrow (○) and the Thy-1° Lin− (□), and Thy-1° Lin+ (△) bone marrow fractions by intravenous (IV) (A and B) or intrathymic (IT) (C and D) transfer techniques. Details of the experiment are given in the text. In A and B, (○) 10^7 syngeneic bone marrow cells were transferred intravenously 4 h after the initial transfer of congenic and syngeneic bone marrow cells, while (□) indicates that the second transfer was not given. Limiting dilution statistics used the average of these two data points. Frequencies, estimated as the negative inverse of the least squares-derived slope, and coefficients of correlation (r values), were as follows: bone marrow IV, 1/36,000 (r = −0.99), IT, 1/5,100 (r = −0.90). Thy-1° Lin− IV, 1/600 (r = −0.96), IT, 1/900 (r = −0.98). Thy-1° Lin+ IV, 1/21,000 (r = −0.95), IT, 1/2600 (r = −0.96). The following data points are not shown on these plots: 1,000 Thy-1° Lin− cells IV, 0% negative animals (8/8+), 400 Thy-1° Lin− cells IT, 0% negative animals (9/9+), 10^4 Thy-1° Lin+ cells IV, 56% negative animals (4/9+), 2,500 Thy-1° Lin+ cells IT, 33% negative animals (6/9+). The limiting dilution statistics include only the latter two of these four data points. Frequencies can also be estimated by interpolating to the x intercept of 37% negative animals. By this method, the frequencies are: bone marrow IV, 1/39,000, IT, 1/8,400. Thy-1° Lin− IV, 1/670, IT, 1/100. Thy-1° Lin+ IV, 1/17,000, IT, 1/2100.

Thy-1° Lin− and Thy-1° Lin+ fractions, the populations were isolated for direct intrathymic injections. This technique has previously been used to examine thymic progenitor cells (14–17). To compare the thymic cloning efficiencies of both fractions with whole bone marrow by both intrathymic and intravenous routes of injection, the following experiment was designed. Two groups, each consisting of 40 C57BL/Ka mice (Thy1.2, Ly1.2, Ly5.1) served as recipients. For intravenous transfers, mice were irradiated (900 rad, 24 h before reconstitution) and reconstituted with 2.5 × 10^6 syngeneic bone marrow cells plus titrated amounts of whole bone marrow from B/6-Ly1.1 donors (Thy1.2, Ly1.1, Ly5.1; 8 × 10^5–5 × 10^4 cells), Thy-1° Lin− cells from BA donors (Thy1.1, Ly1.2, Ly5.2; 150–1,000 cells), and Thy-1° Lin+ cells from B/6-Ly5.2 donors (Thy1.2, Ly1.2, Ly5.2; 800–5,000 cells). For intrathymic transfers, mice were irradiated (800 rad, 3 h before reconstitution) and reconstituted with 10^6 syngeneic bone marrow cells intravenously. The intrathymically injected cell suspensions consisted of a reciprocal titration of syngeneic and B/6-Ly1.1 bone marrow, such that each thymus received 5 × 10^4 bone marrow cells with a titration of congenic marrow in the range of 5 × 10^3–2 × 10^4 cells. In addition, the suspensions contained titrations of BA-derived Thy-1° Lin− cells (50–300 cells) and B/6-Ly5.2-derived Thy-1° Lin+ cells (40–500 cells). This experimental design provides that nearly identical numbers of cells are injected into each animal. Each dose of cells in each titration was injected into eight recipient animals.

FIGURE 4. Detection of donor-derived cells by immunohistochemical analysis. Thymus tissue was analyzed 4 wk after intrathymic injection of 50 FACS-isolated Thy-1° Lin− cells (BA origin) and 4 × 10^5 unseparated bone marrow cells (B/6-Ly5.2 origin) into one thymic lobe of an irradiated (650 rad) C57BL/Ka mouse. The figure shows serial sections stained with anti-Thy-1.1 (A) or anti-Ly-5.2 (B). The uninjected thymic lobe is negative for both stains, while the injected lobe contains progeny of both injected populations. (4X).
Recipient animals were killed 4 wk after intrathymic injection and 5 wk after intravenous injection. Suspensions of thymocytes were screened by FACS for progeny of each donor population, based on expression of the unique allotypic cells surface markers. The results were tabulated as the number of animals in each group that contained progeny of a particular transferred population and are presented as a limiting dilution analysis in Fig. 3. The estimated frequencies of CFU, activity in each fraction as shown in the figure are based on the negative inverse of the slope of each line. The estimates by interpolation to the 37% negative point are indicated in the legend to Fig. 3. The best-fit lines and coefficients of correlation (r) were calculated by least-squares linear regression analysis. The results indicate that whole bone marrow, Thy-1<sup>+</sup> Lin<sup>-</sup>, and Thy-1<sup>-</sup> Lin<sup>+</sup> cells all exhibit a 7–8-fold enhancement of thymic-cloning efficiency when the cell transfer is intrathymic compared with intravenous. The average clone sizes in millions of cells per thymus ranged from 1.1 to 9.8 for intrathymic transfer, and from 2.4 to 20.8 for intravenous transfer. The frequency of CFU, was calculated from the slopes as 1/90 Thy-1<sup>+</sup> Lin<sup>-</sup> cells, 1/2,600 Thy-1<sup>+</sup> Lin<sup>+</sup> cells, and 1/5,100 bone marrow cells by intrathymic transfer. Thus, in this experiment, thymic clone-forming cells were enriched ~60-fold in the Thy-1<sup>+</sup> Lin<sup>-</sup> fraction.

It is of interest to note that only a fraction (1/90) of the Thy-1<sup>+</sup> Lin<sup>-</sup> cells produced thymic colonies after intrathymic injection. It is plausible that in the absence of other bone marrow cells, the thymus is incapable of supporting the survival of many of the directly injected Thy-1<sup>+</sup> Lin<sup>-</sup> cells. This was tested by mixing 50 FACS-isolated Thy-1<sup>+</sup> Lin<sup>-</sup> cells derived from BA mice (Thy-1.1, Ly5.1) with 4 × 10<sup>5</sup> unseparated bone marrow derived from B/6-Ly5.2 mice (Thy-1.2, Ly5.2) and injecting the mixture intrathymically into C57BL/Ka mice (Thy-1.2, Ly5.1). With this experimental design, the unfractionated bone marrow cells might provide the necessary microenvironment and promote better survival of the isolated Thy-1<sup>+</sup> Lin<sup>-</sup> cells after intrathymic transfer. In addition, the B/6-Ly5.2 bone marrow serves as an internal control for the efficiency of the intrathymic injections. The results showed that although nine of nine experimental animals had large populations (5–10%) of Ly5.2<sup>+</sup> cells in the injected thymic lobe, only three of nine animals had detectable levels of Thy-1.1<sup>+</sup> thymocytes (Fig. 4). This data is in good agreement with that shown in Fig. 3, indicating that the coinjected bone marrow did not contribute significantly to the survival of the injected Thy-1<sup>+</sup> Lin<sup>-</sup> cells.

Discussion

The Thy-1 antigen is not readily detectable on non-T cells in normal mouse bone marrow by conventional methods. Mouse bone marrow contains ~5% T cells as determined by immunofluorescence using antibodies specific for Thy-1, CD4, and CD8 (5, 18). Early attempts to eliminate myeloid/erythroid precursors (19) or T cell precursors (20) by treating mouse bone marrow with anti-Thy-1 antibodies and complement failed, indicating that hematolymphoid precursors in the mouse do not express high levels of Thy-1. Recently, however, Thy-1 has been detected on a variety of hematopoietic precursors in mouse bone marrow.
Basch and Berman (21) demonstrated Thy-1 on thymus-homing T cell precursors, and on late (and to a lesser extent on early) myeloid/erythroid precursors, as detected in the CFU assay. They showed that T cell precursors and CFU could be eliminated by anti-Thy-1 antibodies and complement, and CFU could be enriched by FACS. However, they used a highly amplified staining system that indicated Thy-1 expression on 25–30% of mouse bone marrow, including mature B cells and eosinophils. Therefore, this system precluded appreciable enrichment. In a similar amplified stainingsystem, Thy-1 has also been reported to be present on myeloid/erythroid and high proliferative macrophage precursor cells that give rise to in vitro colonies upon stimulation with supernatant derived from mitogen-stimulated spleen cells (22). Complement-mediated cytoxicity was used to demonstrate Thy-1 expression on murine erythroid and myeloid progenitors (23).

We have recently described the isolation of Thy-1-expressing hematopoietic stem cells that completely repopulated the hematolymphoid system of lethally irradiated hosts (5). In our laboratory, Thy-1 is found on only 3–6% of mouse bone marrow cells, with the Thy$^{b}$ Lin$^{-}$ cells contained within 0.1–0.3% of the total population. Thus, we can localize the pluripotent hematopoietic stem cell activity within a very small fraction of mouse bone marrow cells and demonstrate that the precursors of B lymphocytes and myeloid/erythroid lineages (5) and T lymphocytes (this study) are all highly enriched within one fraction of mouse bone marrow.

The frequency of clonogenic CFU$\text{c}$ cells in unfractionated bone marrow is quite low ($\sim$1/3.3 X 10$^{4}$) in the intravenous limiting dilution assay, $\sim$10-fold lower than the frequency of thymus-homing bone marrow cells which upon thymus entry express T cell differentiation markers (20). In contrast, the frequency reported here is at least fivefold higher than that of others (15, 16). This discrepancy is most likely due to a higher inherent cloning efficiency afforded by the true limiting dilution approach used in the present experiments (see reference 12 for a discussion). It is this discrepancy that is responsible for our estimate of the thymic homing efficiency as a factor of 7–8 as compared with 25–50 in other studies (15, 16), since our frequency estimate of CFU$\text{c}$, by intrathymic transfer of whole bone marrow is in good agreement with that obtained by other investigators (Tatsuo Kina, Stanford University, Stanford, CA, personal communication).

A low and variable amount of enrichment for CFU$\text{c}$ has been observed in the Thy-1$^{b}$ Lin$^{-}$ fraction of bone marrow, which might suggest that the activity in this fraction is a result of inefficient FACS separation. More recent experiments (to be reported separately) tend not to support this interpretation, as the Thy-1$^{b}$ Lin$^{-}$ fraction has now been subdivided into three populations, only one of which possesses thymic-homing CFU$\text{c}$ activity (Heimfeld et al., manuscript in preparation). The Thy-1$^{b}$ Lin$^{-}$ population used in these experiments is almost certainly contaminated with Thy-1$^{b}$ Lin$^{+}$ cells, which are in hematopoietic lineages for which we have no markers (e.g., erythroid and megakaryocytic). Recently, the Thy-1$^{b}$ Lin$^{-}$ fraction has been subdivided into two populations based on staining with a newly derived mAb (E13 161-7, see reference 24), and CFU$\text{c}$ activity as well as pluripotent hematopoietic stem cell activity are solely
contained in one of these two populations (Spangrude et al., manuscript in preparation). These observations tend not to support the suggestion that inefficient FACS separation is responsible for the CFU<sub>t</sub> activity in the Thy-1<sup>lo</sup> Lin<sup>+</sup> fraction of bone marrow.

Based on the frequency analysis shown in Fig. 3, one can calculate the percentage of bone marrow CFU<sub>t</sub> activity that can be accounted for by the two FACS-isolated fractions. Using the frequency estimates given by the 37% intercepts (Fig. 3, legend), whole bone marrow contains 12 CFU<sub>t</sub>/10<sup>5</sup> cells injected intrathymically. The Thy-1<sup>lo</sup> Lin<sup>-</sup> fraction, comprising 0.25% of the total bone marrow, contains 1 CFU<sub>t</sub>/100 cells and thus contributes 2.5 CFU<sub>t</sub>/10<sup>5</sup> bone marrow cells. Correction of this number to compensate for the purity of the sorted fraction as measured by reanalysis (5, 6) allows an estimate of 4.2 CFU<sub>t</sub>/10<sup>5</sup> bone marrow cells being contributed by the Thy-1<sup>lo</sup> Lin<sup>-</sup> fraction. A similar calculation for the Thy-1<sup>lo</sup> Lin<sup>+</sup> fraction, which comprised 4.6% of the total bone marrow in the experiment shown in Fig. 3, results in an estimate of 2.2 CFU<sub>t</sub>/10<sup>5</sup> bone marrow cells being derived from this fraction, with no correction for the purity of the sorted fraction. Thus, by a very conservative estimate, which assumes that cellular functions and potentials in the sorted fractions are unaffected by a 4–5h staining and cell-sorting regimen, one can account for over half of the CFU<sub>t</sub> activity in whole bone marrow as being recovered in the two Thy-1<sup>lo</sup> fractions. In addition, some CFU<sub>t</sub> activity was detected by intrathymic transfer of the cells in bone marrow fraction II (as defined in Fig. 2), but this activity was less than that in unseparated bone marrow (data not shown) and failed to home to the thymus (Table 1).

It has been a generally accepted concept that the pluripotent hematopoietic stem cell in the bone marrow makes a commitment to the lymphoid lineage (25) or even to the T lymphoid lineage (26, 27) before homing to the thymus, where further differentiation occurs. Cloned bone marrow cell lines have been described (28) that have the properties of progenitor cells that are committed to the T lineage. Although Mulder et al. (29) have presented evidence supporting the existence of bone marrow pre-T activity, which by kinetic analysis is due to a more mature progenitor than the pluripotent hematopoietic stem cells, they failed to demonstrate whether this population of cells is solely committed to differentiation along the T lineage. Both fractions of bone marrow characterized herein, as well as subsequent subfractions of those populations identified by expression of combinations of antigenic determinants, contain CFU<sub>t</sub> only in the presence of self-renewing pluripotent hematopoietic stem cells (contained in the Thy-1<sup>lo</sup> Lin<sup>-</sup> fraction) or of precursors of multiple lineages (contained in the Thy-1<sup>lo</sup> Lin<sup>+</sup> fraction) (data not shown). Whether committed bone marrow prothymocytes can be separated from multipotent precursors by cell-sorting methods remains to be elucidated. Alternatively, the final commitment to maturation along the T lineage may occur only after a multipotent progenitor cell enters the thymus. According to the latter view, hypothesized by Moore and Owen (30), the only true prothymocytes will reside solely within the thymus.

The maturational development of a functional immune system is of considerable interest, in both experimental and clinical applications. It is important to define the process of immune maturation in ontogeny as well as in the recovery
of the immune system from radiation, from chemotherapy of malignancies, and from immunosuppressive viral diseases such as AIDS. Further, insight into immune maturation can help define specific lesions in the processes that result in various genetically acquired or congenital immune deficiencies. Finally, the isolation of highly enriched, and perhaps pure populations of hematopoietic stem cells shown in this and previous communications (6,31), should be valuable for bone marrow allotransplantation, for gene transfection experiments, and ultimately, for a complete understanding of the process of hematolymphoid development.

Summary

Two-color FACS analysis of mouse bone marrow reveals a rare population, comprising 0.1–0.3% of the total, that expresses low levels of the Thy-1 antigen but does not express any of five surface markers that characterize differentiated hematolymphoid cells. We demonstrate here that this fraction of mouse bone marrow is enormously enriched in cells that can home to the thymus and differentiate into mature T lymphocytes, subsequently migrating to peripheral lymphoid organs. Only a subset of the FACS-isolated fraction (1/90 after intrathymic injection) is capable of responding to the thymic microenvironment with a productive commitment to the T cell lineage. A second fraction of mouse bone marrow, which expresses how levels of Thy-1 but is also positive for at least one of five hematolymphoid lineage-specific markers, also contains cells that home to the thymus and establish colonies of thymocytes. The two fractions each contribute approximately equal amounts of thymic colony-forming units (CFU) to the bone marrow, and together can account for at least half of the CFU, in whole bone marrow.

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