Developmental Potential of the Earliest Precursor Cells from the Adult Mouse Thymus
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Summary
A new, numerically minute population of cells representing the earliest T precursor cells in the adult mouse thymus has recently been isolated. This population has been shown to be similar to bone marrow hemopoietic stem cells in surface antigenic phenotype and to express moderate levels of CD4. We now show, by fluorescence-activated cell sorting and intrathympic transfer to irradiated mice, that this apparently homogeneous population differs from multipotent stem cells in expressing the surface stem cell antigen 2 (Sca-2), that it differs from most early B lineage cells in lacking B220 and class II major histocompatibility complex expression, and that it binds rhodamine 123 like an activated rather than a quiescent cell. Irradiated recipient mice differing at the Ly 5 locus were used to compare the developmental potential of these early intrathympic precursors with bone marrow stem cells. Only T lineage product cells were detected when the intrathympic precursor population was transferred back into an irradiated thymus. However, when the intrathympic precursor population was transferred intravenously, it displayed the capacity to develop into both B and T lymphoid cells in recipient bone marrow, spleen, and lymph nodes, but no donor-derived myeloid cells were detected. The absence of myeloid and erythroid precursor activity was confirmed by showing that the intrathympic precursor population was unable to develop into myeloid or erythroid spleen colonies on intravenous transfer or to form colonies in an agar culture. These findings indicate that this earliest intrathympic precursor population has become restricted (or strongly biased) to lymphoid lineage development, but not exclusively to T lymphocytes.

The ultimate origin of T lymphocytes is the same as that of other hemopoietic cells, namely a multipotent stem cell found in fetal liver and adult bone marrow (1–3). A continuous but low input from bone marrow of some form of stem cell or “prothymocyte” appears necessary to maintain T cell development in the adult thymus (4). A prothymocyte–like population, of which virtually every cell was able to colonize an irradiated thymus on intrathympic transfer, has been purified from adult mouse bone marrow (5, 6). Since each cell of this kind was also able to reconstitute erythroid, myeloid, and B lymphoid lineages, this precursor was a form of multipotent hemopoietic stem cell. This purified bone marrow stem cell expressed low levels of Thy-1 but had no detectable expression of other hemopoietic lineage markers; it was positive for stem cell antigen-1 (Sca-1;1 Ly 6A/E), Pgp-1 (CD44), heat stable antigen (HSA), and MHC class I. However, it is not clear whether this is the cell that normally migrates through the blood to colonize the thymus. In attempting to clarify this issue, it is important to identify the earliest intrathympic precursors that might represent a point of connection with the cells seeding the thymus from bone marrow and to test their developmental capacity.

The earliest intrathympic precursor population so far observed in the adult thymus has been isolated recently in this laboratory as a minute population that expressed moderate levels of mature T cell marker CD4 (7). This population, termed the “low CD4 precursor,” resembled the bone marrow hemopoietic stem cell in surface antigenic phenotype (Thy-1+, HSA+, Pgp-1+, H-2K2+), and Sca-1+) and had TCR and Ig genes in germline state. On intrathympic transfer to irradiated animals this low CD4 precursor population showed slower reconstitution kinetics and higher expansion potential than the CD48+ thymocytes, previously considered the earliest precursors (8). The T cells produced included both the TCRαβ and -γδ T lineages (7, 9). These findings suggest that cells within the low CD4 precursor population were

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1 Abbreviations used in this paper: HSA, heat-stable antigen; Lin− BM, lineage marker-negative bone marrow; Rh-123, rhodamine 123; Sca, stem cell antigen.
the immediate progeny of a thymus-seeding bone marrow stem cell.

Since this intrathymic low CD4 precursor shared many properties with bone marrow stem cells, it was important to ask whether it was also multipotent. To answer this question, we transferred the low CD4 precursors intravenously to irradiated recipients and so compared their developmental potential with that of bone marrow stem cells. Our studies demonstrate that the earliest intrathymic precursor cells differ from bone marrow stem cells, having lost the capacity to develop into erythroid and myeloid cells and having become restricted or strongly biased to lymphoid lineage development.

Materials and Methods

Mice. Mice of C57BL/Ka Thy-1.2, C57BL/Ka Thy-1.1, and C57BL/6-Ly 5.1-Pep strains were bred at The Walter and Eliza Hall Institute Animal Facility. For intrathyrmic T lineage reconstitution experiments, C57BL/Ka Thy-1.2 (Ly 5.2) and the Thy-1-congenic C57BL/Ka Thy-1.1 (Ly 5.2) mice were used interchangeably as donors and recipients (4-6 wk of age as donors or 8-12 wk of age as recipients). For hematopoietic lineage reconstitution, C57BL/Ka Thy-1.1 (Ly 5.2) mice 4-6 wk of age were used as donors, and C57BL/6-Ly 5.1-Pep (Thy-1.2, Ly 5.1) mice 8-12 wk of age were used as recipients.

Monoclonal Antibodies and Fluorescent Reagents. The antibodies used for cytotoxic destruction were: anti-CD8, clone H0-2.2 (10); anti-CD3, clone 17A2 (11); and anti-IL-2Rα, clone 7D4 (12). The antibodies used for magnetic bead depletion were: anti-CD8, clone 53-6.7 (13); anti-CD3, clone KT3-1.1 (14); anti-IL-2R, clone PC61 (15); anti-CD2, clone RM2-1 (16); anti-B220, clone RA3-6B2 (17); anti-Mac-1, clone M1/70.15 (18); anti-Gr-1, clone RB6-8C5 (19); and anti-erythrocyte antigen, clone TER-119 (derived by Tatsuo Kina and colleagues, Department of Immunology, Chest Disease Research Institute, Kyoto University, Kyoto, Japan). All the above antibodies used for immunofluorescent staining were: anti-Tby-l.1, clone 19F-12 (20), used as a direct FITC conjugate; anti-HSA, clone 53-6.7 (13); anti-Thy-1, clone 17A2 (11); and anti-H-2Kb, clone B8-24-3 (21), used as a biotinylated conjugate. The fluorescent reagents used for two- and three-color fluorescent staining procedures were similar to those described previously (7,22). Flow cytometric analysis or sorting was performed on FACStar Plus™ (Becton Dickinson & Co., Mountain View, CA).

Flow Cytometric Staining and Cell Sorting. Two- and three-color fluorescent staining procedures were similar to those described previously (7,22). Flow cytometric analysis or sorting was performed on FACStar Plus™ (Becton Dickinson & Co., Mountain View, CA). Dead cells were gated out on the basis of forward light scatter and balanced salt solution for direct macroscopic surface counts of spleen colonies (stained with FITC-conjugated antibodies; anti-Thy-1, clone 17A2 (11); and anti-H-2Kb, clone B8-24-3 (21), used as a biotinylated conjugate). The fluorescent reagents used for two- and three-color fluorescent staining procedures were similar to those described previously (7,22). Flow cytometric analysis or sorting was performed on FACStar Plus™ (Becton Dickinson & Co., Mountain View, CA). Dead cells were gated out on the basis of forward light scatter and balanced salt solution for direct macroscopic surface counts of spleen colonies (stained with FITC-conjugated antibodies; anti-Thy-1, clone 17A2 (11); and anti-H-2Kb, clone B8-24-3 (21), used as a biotinylated conjugate).

Isolation of Low CD4 Intrathymic Precursor Cells. The procedure was basically as described previously (7). Thymocyte suspensions from 16-24 thymuses of 4-6-wk-old C57BL/Ka mice were subjected to two successive depletion procedures to remove CD4+ 8+ cortical thymocytes, CD3+ mature medullary thymocytes, most of the CD4+ 8+ precursor cells, and non-T lineage cells, all without depletion on the basis of CD4 expression. The first step was complement-mediated cytotoxicity using antibodies against CD4, CD8, and IL-2Rα. Dead cells were then removed by metrizamide density cut. In some experiments, where noted, adherent macrophage-like cells were depleted at this stage by incubating cells (suspended in RPMI 1640 containing 10% FCS) in a plastic petri dish at 37°C for 90 min. The nonadherent cells were collected by gently washing the dish three times with warm medium. Residual contaminants and further unwanted cells were removed by coating the cells with anti-CD8, anti-CD3, anti-IL-2Rα, anti-CD2, and with antibodies against erythrocytes (TER-119), granulocytes (anti-Gr-1), macrophages (anti-Mac-1), and B cells (anti-B220 and anti-Ig), then removing the coated cells with anti-Ig-coated magnetic beads (using an 8:1 ratio of beads to cells and a mixture of three parts anti-rat Ig and one part anti-mouse Ig Dynabeads [Dynal Inc., Oslo, Norway]). In some experiments, where noted, depletion of B220-bearing cells was omitted. The fully depleted population, now representing  <1% of thymocytes, was stained in three fluorescent colors with PE-anti-HSA, FITC-anti-Thy-1, and biotin-anti-H-2Kb, and biotin-anti-H-2Kb, followed by staining with Texas red-avidin. The stained cells were then sorted for cells expressing low but positive levels of Thy-1, moderate levels of HSA, and very high levels of H-2K. All these Thy-1+, HSA-, and H-2K+ cells have been shown previously to express moderate levels of CD4 (7), although this was not used as a sorting parameter in the present study. Reanalysis showed that the purity of the sorted population was >97%.

Preparation of Low Thy-1, Low HSA, and Lineage Marker-negative Bone Marrow Cells (Lin- BM). This preparation represented an enriched source of hemopoietic stem cells, depleted of most lineage-committed precursors. Bone marrow cells were obtained from adult C57BL/Ka Thy-1.1 mice. Erythrocytes and dead cells were removed as previously described (23). Lineage marker-negative bone marrow cells were then depleted with antibodies against macrophages (anti-Mac-1), granulocytes (anti-Gr-1), erythrocytes (TER-119), and B lymphocytes (anti-B220), followed by anti-Ig-coated magnetic beads. The depleted cells were stained in three colors in the following sequence: (a) cells were coated with the same antibodies used for magnetic bead depletion, followed by PE-conjugated goat anti-rat Ig as second stage; (b) this second stage was then blocked with rat Ig; (c) the cells were then stained simultaneously with FITC-anti-Thy-1.1 and APC-anti-HSA. The Lin- BM cells were obtained by sorting for cells that were negative for lineage markers and low positive for both Thy-1 and HSA.

Thymic Reconstitution Assay. The procedure has been described in detail (24). The Thy-1 congenic recipient mice were irradiated (7.5 Gy γ irradiation) and used 1-3 h later. The cells in 10 μl of balanced salt solution were injected directly into a single lobe of the recipient thymus. At various times after transfer, cell suspensions were prepared from the injected recipient thymus lobes and stained with FITC-conjugated antibodies against the donor-type Thy-1 (in some experiments Ly 5), together with anti-CD4 (PE-conjugated) and anti-CD8 (biotinylated, with Co-Av second stage). The stained cells were then analyzed by flow cytometry, gating for Thy-1+ donor-derived cells.

Spleen Colony Forming Assay. The procedure was similar to that of Till et al. (25) and Molineux et al. (26). The recipient mice were given two doses of 5.5 Gy γ irradiation with a 3-h interval between, then used ~1 h later. Various numbers of the test precursor cells were injected intravenously. To help maintain the mice, neonatain sulfate (1.1 g/liter) was included in the drinking water. The spleens were removed 8 or 12 d after transfer and fixed in Bouin’s solution for direct macroscopic surface counts of spleen colonies or for sectioning for histological examination.

Lineage Reconstitution Assay Using the Ly 5 Marker. The procedure was similar to that of Spangrude et al. (6). The Ly 5.1 recip-
ient mice were lethally irradiated (two doses of 5.5 Gy γ irradiation with a 3-h interval between) and then used <1 h later. The donor cells from Ly 5.2 mice were injected intravenously, along with 4 × 10^4 Ly 5.1 (recipient type) unfractionated bone marrow cells, to ensure long-term survival of the recipients. Antibiotics (polymyxin B sulfate, 10^5 U/liter; neomycin sulfate, 1.1 g/liter) were added to the drinking water. 2–8 wk after transfer, the spleen and lymph nodes (pooled), the thymus, and the bone marrow were collected from recipients. Cells were stained in two colors with donor-specific anti-Ly 5.2 (FITC-conjugated) and biotinylated lineage-specific antibodies (either anti-Thy-1.1, anti-B220, anti-Mac-1, or anti-Gr-1), followed by PE-avidin for the second stage. In some experiments, where noted, three-color staining was used for analysis. In such experiments cell suspensions were stained with PE-conjugated anti-mouse Ig, then blocked with mouse Ig, followed by staining with FITC-conjugated Ly 5.2 and biotinylated B220; Texas red-avidin was used for the next stage staining.

**Agar Colony Assay for Myeloid Precursors.** The semi-solid agar cultures were performed according to the procedures described by Metcalf et al. (27). Briefly, equal volumes of double-strength culture medium (DME-20% FCS) and double-strength agar (0.6%) were mixed at 37ºC. The cells to be cultured were added, and 1 ml of the cell suspension in the agar medium was pipetted into 35-mm petri dishes containing the stimulus required for the proliferation of myeloid precursors. After mixing, the cultures were allowed to gel and then were incubated at 37ºC in 10% CO₂, 90% air. The colonies were scored 7–14 d later. The stimulating factors used in this study were recombinant stem cell factor (27a) (200 ng/ml), IL-1 (100 U/ml), IL-3 (1,000 U/ml), M-CSF (1,000 U/ml), G-CSF (1,000 U/ml), GM-CSF (1,000 U/ml), and erythropoietin (EPO) (1 U/ml).

**Preparation for Light and Electron Microscopy.** For light microscopy 40,000 purified cells were cytospun onto a glass slide, fixed in 89% methanol, 10% distilled water, and 1% acetic acid, and stained with Giemsa. For electron microscopy, the cells were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2), then postfixed for 1 h at room temperature in 2% OsO₄. The fixed cells were stained en bloc with 4% uranyl acetate (aqua) at 4ºC in the dark and dehydrated in graded acetones (10–100%). The sample was infiltrated with Spurr resin, polymerized and sectioned, stained with 10% uranyl acetate for 5 min and then with Reynolds's lead citrate for 5 min. The stained sample was viewed on an electron microscope (model CM12; Philips Electronic Instruments Co., Mahwah, NJ) at 60 kV.

**Results**

**Sca-1⁺ Sca-2⁺ Fraction of the Low CD4 Precursor Population Contains Early Precursor Activity.** Sca-1 (Ly 6A/E) and Sca-2 have been used to characterize stem cells and precursor cells from bone marrow (5, 28). To test the Sca-1 and Sca-2 expression of the earliest intrathymic precursor cells, the depleted, CD8⁻, CD3⁻, CD2 -, and IL-2R⁻, and of non-T lineage marker-negative thymocytes were stained in four colors with antibodies to Thy-1.1, HSA, Sca-1, and Sca-2; the stained cells were then analyzed by flow cytometry. The staining pattern is shown in Fig. 1. The low CD4 precursor population stood out distinctly with low levels of Thy-1 and moderate levels of HSA (Fig. 1 A). Our previous studies (7) showed that these cells (within the gates shown in Fig. 1 A) all expressed moderate levels of CD4, high levels of Pgp-1, and very high levels of H-2K, and included all the early thymocyte precursor activity of the depleted preparation. Separate experiments also established that these cells were MHC class II (Ia)⁻negative or very low positive (data not shown). When this gated precursor population was analyzed, it was clear that most cells (>90%) were positive for expression of both Sca-1 (Fig. 1 B) and Sca-2 (Fig. 1 C). Further analysis (not shown) indicated that these two markers were tightly correlated, with most of the 5% Sca-2⁻ cells also being Sca-1⁻.

**Figure 1.** Expression of Sca-1 and Sca-2 on the thymic low CD4 precursor population. Thymocytes were depleted of cells bearing CD3, CD8, CD2, and IL-2R, and of non-T lineage cells (see Materials and Methods). The depleted population (<1% thymocytes) was stained in three colors with antibodies against Thy-1 (FITC-19F-12), HSA (PE-M1/69), and Sca-1 (biotin-B3 161-7) or Sca-2 (biotin-B3 81-2). (A) HSA and Thy-1 distribution, with the area previously shown to include all early T precursor cells (7) bound by the broken line. (B) Distribution of Sca-1, and (C) the distribution of Sca-2 on this gated population with the background fluorescence is shown by a dotted line. One of two similar analyses is presented.

**Figure 2.** Thymus reconstitution activity of the Sca-2⁺ and Sca-2⁻ fractions from the low CD4 precursor population. The low CD4 precursor population was isolated and stained as in Fig. 1 C, then sorted for cells that were Thy-1 low, HSA intermediate (using the gates of Fig. 1 A), and either Sca-2⁺ or Sca-2⁻ (according to the distribution in Fig. 1 C). The sorted fractions were then injected into the thymus of irradiated Thy-1 congenic mice (see Materials and Methods) using 9,000 cells per lobe for the major Sca-2⁺ population, and 1,800 cells per lobe for the minor Sca-2⁻ population. At the times stated, suspensions of the pooled recipient thymus lobes were analyzed for cells of donor Thy-1 type (Thy-1⁺). No donor-derived cells were seen when the Sca-2⁻ cells were injected. One of two similar experiments is presented.
As described by Spangrude et al. (5, 29), purified bone marrow stem cells are Sca-1+ but Sca-2-. To test whether the precursor activity of our low CD4 precursor population was derived from the majority of cells that express Sca-2 or from a small minority of Sca-2- cells that could possibly be bone marrow-derived stem cells, we separated Sca-2+ and Sca-2- cells by sorting and then examined their thymic reconstitution ability after intrathymic injection. As shown in Fig. 2, 9,000 Sca-2+ cells when transferred, produced 20-30% donor-derived cells at 14 d and 50-60% donor-derived cells at 23 d, a result similar to that obtained with the total low CD4 precursor population. In contrast, no donor-derived cells were detected at either time after transfer of 1,800 Sca-2- cells, even though this number was large in proportion to their representation within the low CD4 precursor population. The T precursor activity was evidently within the major Sca-2+ subpopulation and was not derived from a small number of Sca-2- cells resembling bone marrow stem cells.

The B220- Fraction of the Low CD4 Precursor Population Contains the Early Precursor Activity. In preliminary isolation protocols where anti-B220 was omitted from the depletion procedure (any thymic B cells being removed by the anti-Ig-coated beads), the Thy-1-low, HSA-intermediate, and CD4-intermediate fraction included a 10-15% subpopulation of small-sized B220- cells (Fig. 3). In view of the finding by Strasser et al. (30) of a stem-cell-like tumor cell bearing both CD4 and B220, the precursor function of the B220- subset of our thymic low CD4 precursor preparation was assessed. Both the B220- (major subpopulation) and the B220+ (minor subpopulation) cells were isolated as in Fig. 3, then transferred intrathymically to test for thymus reconstitution activity. All the activity of the Thy-1-low, HSA-intermediate, low CD4 precursor population could be accounted for by the major B220- subpopulation, with no activity being detected from the B220+ cells (data not shown). Accordingly, in most subsequent experiments this B220- subpopulation was removed from the preparation by including anti-B220 in the depletion procedure.

The Fraction of the Low CD4 Precursor Population Staining Brightly with Rhodamine Contains the Early Precursor Activity. Rhodamine 123 (Rh-123) is a fluorescent vital dye that preferentially binds to mitochondrial membranes in living cells (31), the intensity of staining being related not only to the number of mitochondria per cell but also to the state of cellular activation (32). This dye has been used to separate activated from quiescent stem cells (33, 34). When the thymic low CD4 precursor population was stained with Rh-123, most
cells (95%) stained very brightly (results not shown). The 95% Rh-123<sup>hi</sup> cells were separated by sorting from the 5% Rh-123<sup>lo + med</sup>, and thymus reconstitution activity was assessed in experiments similar to those shown in Fig. 2. The Rh-123<sup>hi</sup> fraction showed high thymic reconstitution activity, whereas no reconstitution was seen in the Rh-123<sup>lo + med</sup> cells (data not shown). Thus, the activity of the low CD4 precursor population resided in the majority population of bright rhodamine staining, metabolically active cells.

**Morphological Appearance of the Low CD4 Precursor Population.** The low CD4 precursor population was purified using the full procedure, including depletion of B220<sup>+</sup> cells and adherent cells. The general appearance of these preparations (Fig. 4, A and B) was of cells intermediate between lymphoblasts and purified hematopoietic stem cells (5, 35). Mitotic figures were seen, in accordance with our previous data indicating that 14% of the cells were in S phase (7). Mitochondria were plentiful, in accordance with the rhodamine staining data and the concept that the low CD4 precursors represent an active rather than a quiescent population.

**The Low CD4 Precursor Does Not Contain CFU Activity.** Because of the similarities in surface antigenic phenotype between the thymic low CD4 precursor cells and bone marrow stem cells, it was important to determine whether the thymic precursor cells were also multipotent. As a first test we checked their ability to form erythroid and myeloid colonies in the spleen, the standard CFU assay. When purified thymic low CD4 precursor cells were transferred intravenously into lethally irradiated recipients, no spleen colonies were found after 8 d, and after 12 d an average of only two colonies were found per 30,000 cells injected (Table 1). Compared with purified bone marrow stem cells (5), the thymic low CD4 precursor population was, on a cell for cell basis, 1,000-fold less effective at forming spleen colonies. To ensure that this lack of CFU capacity was not due simply to the antibodies on the cell surface preventing seeding in the spleen (36), the depleted thymocyte preparation was also tested for CFU activity before coating the cells with antibodies for positive sorting. As shown in Table 1, this depleted preparation did not contain significant CFU activity. Thus, by the criterion of the CFU assay, the low CD4 precursor cells were unable to serve as precursors of the erythroid or myeloid lineages.

**The Low CD4 Precursor Population Contains Few Cells Capable of Forming Myeloid or Erythroid Colonies in Agar.** As a parallel to the CFU tests, we checked whether the low CD4 precursor population was able to form colonies in agar in the presence of growth factors capable of inducing precursor cells to develop into granulocytes, macrophages, or erythroid cells. In initial studies where depletion of adherent cells was not used, a mean of 7% of the low CD4 precursor population formed small clusters (<50 cells) and an occasional colony consisting entirely of macrophages, with no polymorphonuclear or erythroid cells. This observation confirmed that no multipotent cells were present, but suggested the presence of some committed, late macrophage precursors in the preparation, despite the Mac-1 depletion step and the verified absence of Mac-1<sup>+</sup> cells. Accordingly, a period of incubation on plastic dishes was introduced into the isolation protocol to remove possible macrophage precursors. Only a small proportion of the cells was removed by this process, and average cell recoveries were not detectably reduced. After intrathymic transfer into irradiated Thy-1 congenic recipients, the adherent cell–depleted preparation showed the normal distribution of thymocyte progeny 14 d after transfer, with an apparent expansion in numbers from 680- to 1,180-fold, certainly no lower than the average 500-fold expansion observed with preparations not depleted of adherent cells (7). However, now only between 0 and 0.7% of the cells in the adherent cell–depleted low CD4 precursor preparations gave colonies in agar. These few colonies consisted only of macrophages, with no polymorphs or erythrocyt cells. It was concluded that the few macrophage precursors present were separate from the precursor cells leading to the T lineage.

**Hematopoietic Lineage Reconstitution by the Low CD4 Precursor Population.** To assess further the developmental potential of the thymic low CD4 precursor population, the system of intravenous transfer into mice differing at the Ly 5 locus was used (6). This system allowed even low levels of lineage reconstitution to be analyzed (Fig. 5). This was important since to ensure the long-term survival of the recipient animals, recipient-type bone marrow cells were also transferred, which meant that the thymic precursors were competing with bone marrow stem cells for reconstitution “space.” At weekly intervals 2–8 wk after transfer, we analyzed spleen and lymph

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**Table 1. Spleen Colony Assay (CFUs)**

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<thead>
<tr>
<th>Cell source</th>
<th>No. injected</th>
<th>Colonies examined</th>
<th>Average colonies per spleen</th>
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<tr>
<td>Thymic CD8&lt;sup&gt;-3·2-&lt;/sup&gt;, IL-2R&lt;sup&gt;-&lt;/sup&gt;,Thy-1&lt;sup&gt;+&lt;/sup&gt;, HSA&lt;sup&gt;-&lt;/sup&gt;,H-2K&lt;sup&gt;+&lt;/sup&gt;,(low CD4 precursors)</td>
<td>30,000</td>
<td>8</td>
<td>0</td>
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<tr>
<td></td>
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<td>12</td>
<td>2</td>
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<tr>
<td>Thymic CD8&lt;sup&gt;-3·2-&lt;/sup&gt;, IL-2R&lt;sup&gt;-&lt;/sup&gt; (depleted only)</td>
<td>300,000</td>
<td>12</td>
<td>1</td>
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<tr>
<td>Bone marrow</td>
<td>100,000</td>
<td>8</td>
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<td>Unseparated cells</td>
<td>12</td>
<td>14</td>
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<tr>
<td>Purified bone marrow stem cells</td>
<td>200</td>
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<td>12</td>
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The full details of the spleen colony assay are given in Materials and Methods. Results summarized in this table are the means of two experiments, each involving three recipients per time point. The data for purified bone marrow stem cells are from previously published work (5), presented here for comparison.
low CD4 precursor cells, along with 40,000 unseparated host type bone marrow cells, were intravenously transferred into irradiated mice differing at the Ly 5 locus (see Materials and Methods). 30,000 purified marrow cells, were intravenously transferred into irradiated mice differing donor and host differed in both Ly 5 and Thy-1 allotypes, so host type at the Ly 5 locus (see Materials and Methods). 4 wk after reconstitution, the recipient spleen and lymph nodes (pooled) were analyzed for donor-derived cells by use of two-color flow cytometry with FITC-anti-Ly 5.2 and biotinylated lineage marker antibodies, either anti-Thy-1.1, anti-B220, anti-Mac-1, or anti-Gr-1, with PE-Av as second stage. In this experiment donor and host differed in both Ly 5 and Thy-1 alleles, so host type Thy-1.2+ cells were not stained. The result presented is representative of a series of similar experiments.

In the thymus of recipient mice the maximal level of reconstitution by the low CD4 precursors was obtained 3 wk after intravenous transfer, and it declined rapidly thereafter (Fig. 6 A). This peak of transient reconstitution was similar to that seen with intrathymic transfer (7). The level of reconstitution (30% donor-derived cells) was higher than that seen in other lymphoid organs (compare with Figs. 7 and 8) and was similar to the level obtained with bone marrow stem cells at this time point. However, the reconstitution by Lin- BM cells rose beyond this, reaching a peak at 4 wk, and this high level of reconstitution continued to 8 wk (presumably by continuous seeding from bone marrow). Overall, the results suggested that the low CD4 precursor cells gave a transient rather than a self-renewing pattern of reconstitution and had a low expansion potential as compared with bone marrow stem cells.

The nature of the progeny cells in the recipient thymus was examined at each time point (Fig. 6 B). Only Thy-1+ donor-derived cells were detected from both the low CD4 precursors and the Lin- BM populations. In separate experiments (data not shown), it was verified that at 3 wk the donor-derived cells consisted mainly of CD4+8+ cortical thymocytes, with a small proportion of CD4+8- and CD4-8+ mature thymocytes, as seen previously with intrathymic transfer (7). No detectable level of production of either B cells or myeloid cells was seen (Fig. 6 B). Similar restriction of the low CD4 precursor cells to T cell production was seen in separate experiments when they were injected intrathymically and Ly 5 was used to track the progeny (data not shown).
In recipient bone marrow the level of reconstitution by the low CD4 precursor population was very low at all time points (Fig. 7A). The maximum was ~3% at 3 wk, whereas Lin- BM cells gave 60–70% donor-derived cells. It was nevertheless possible to determine the expression of lineage markers by the cells derived from low CD4 precursors (Fig. 7B). In contrast to the results with Lin- BM, the majority of progeny were Thy-1-bearing cells. This result was obtained even at the 2-wk time point; the nature of these early progeny cells was not further determined. Very few of the progeny were Mac-1+ or Gr-1+, and the small proportions recorded were close to the background limits of analysis. However, the surprising result was that the low CD4 precursors gave a significant proportion of B220+ progeny at all time points, a proportion similar to that obtained with Lin- BM.

In the spleen and lymph nodes, the peak level of reconstitution by the low CD4 precursor population at 4 wk was ~10% (Fig. 8A), being higher than that in bone marrow but lower than that in thymus. However, the reconstitution was more persistent than that observed in thymus or bone marrow. Since in preliminary experiments the overall pattern seemed similar in spleen and lymph nodes, cells from these sources were pooled in the experiments given in Fig. 8. An example of the analysis at a 4-wk time point is given in Fig. 5. As with bone marrow, a high proportion of the progeny of the low CD4 precursors were Thy-1+, even at the earliest time points (Fig. 8B); many of these cells expressed only low levels of Thy-1. Very few of the progeny were Mac-1+ or Gr-1+ (and the levels measured were close to the background for this analysis), in contrast to high incidence of myeloid cells seen at the early time points when Lin- BM was transferred. However, as found in the bone marrow, the B lineage as well as the T lineage was reconstituted by the thymic low CD4 precursor cells, since 20–40% of the progeny were B220+ over the 2–8-wk period (Figs. 7B and 8B).

**Thy-1+ Progeny of the Low CD4 Precursors Are T Cells.** To determine whether the Thy-1+ progeny of the low CD4 precursors were normal T cells, the recipient spleen and lymph node cells were analyzed 3 and 4 wk after transfer by multicolor staining with anti-Ly 5.2, anti-Thy-1, anti-CD4, and anti-CD8. The gated Ly 5.2+ Thy-1+ cells were found to

![Figure 7](https://example.com/fig7.png)  
**Figure 7.** Bone marrow reconstitution by the thymic low CD4 precursor population and Lin- BM cells. The procedures for intravenous transfer, analysis of donor-derived cells, and other details are as described in Fig. 6. (A) Overall reconstitution by both the low CD4 precursor population and the Lin- BM cells in the recipient bone marrow. (B) The lineage distribution of donor-derived cells. Proportions <3% in the lower panels were within the background range.

![Figure 8](https://example.com/fig8.png)  
**Figure 8.** Spleen and lymph node reconstitution by the low CD4 precursor population and Lin- BM cells. The procedures for intravenous reconstitution, analysis of donor-derived cells, and other details are the same as described in Fig. 6. (A) Overall reconstitution by both low CD4 precursors and the Lin- BM cells in the recipient spleen and lymph nodes (pooled). (B) Lineage distribution of donor-derived cells. Proportions <3% in the lower panels were within the background range.
Figure 9. Further analysis of donor-derived B220+ cells in the spleen and lymph nodes of irradiated mice reconstituted with thymic low CD4 precursors. In the same experiment described in Fig. 5, the pooled cell suspension from recipient spleen and lymph nodes was stained in three fluorescent colors, first with PE-conjugated anti-mouse Ig, followed by blocking with mouse Ig, then staining with FITC-conjugated anti-Ly 5.2 and biotinylated B220, using TR-Av as second stage. The stained cells were then analyzed by flow cytometry. The donor-derived Ly 5.2+ cells, or the Ly 5.2+ B220+ cells, were gated for further characterization. (A) B220 and surface Ig distribution on the gated Ly 5.2+ donor-derived cells. (B) Forward light scatter of the gated Ly 5.2+ B220+ cells. The dotted line shows the forward light scatter of normal splenic B220+ cells.

be 65% CD4+8− and 32% CD4−8+ (data not shown), a distribution similar to that of normal peripheral T cells.

The B220+ Progeny of the Low CD4 Precursors Are B Cells. To determine whether the B220+ cells derived from the thymic low CD4 precursors were conventional B cells, they were further analyzed by multicolor staining of recipient spleen and lymph node cells with antibodies to donor Ly 5, B220, and mouse Ig, and by gating during analysis for donor-type Ly 5.2+ cells. The donor-derived B220+ cells were all surface Ig+ and had the light scatter characteristics of normal splenic B lymphocytes (Fig. 9). In separate experiments the B220+ donor-derived cells were stained with anti-CD5 and were found to be predominantly CD5− (data not shown). Thus, most B220+ donor-derived cells appeared to be normal B cells.

B220+ B-Cell Progeny of the Low CD4 Precursors Derive from B220− Cells. To ascertain that the B cells developing from the low CD4 precursor preparation did not derive from some B220+ pre-B cell contaminants, B220 depletion was omitted from the isolation protocol, and the resultant population was stained and sorted into distinct B220+ and B220− components (shown in Fig. 3). The sorted fractions were transferred intravenously into Ly 5 congenic recipients, and 4 wk later the spleen and lymph nodes (pooled), the bone marrow, and the thymus were analyzed for donor-derived cells. Only the B220− fraction showed significant reconstitution activity, the B220+ fraction showing levels close to the background for the system (Table 2). On reconstitution the B220− fraction gave both Thy-1+ and the B220+ donor-derived cells, in spleen and lymph nodes and in bone marrow, but only Thy-1− cells in the thymus. This result is exactly the same as that found for the total low CD4 precursor population. In separate experiments (not shown), similar reconstitution of both the Thy-1+ and the B220+ lineage was obtained with adherent cell-depleted low CD4 precursor populations. Clearly all the T and B precursor activity of the thymic low CD4 precursor preparations resided in the B220− and nonadherent cells, which constitute the bulk of this preparation.

Discussion

Surface Phenotype of Thymic Low CD4 Precursors Compared to Bone Marrow Stem Cells. In this study we have compared the earliest T precursor cells from the adult mouse thymus with bone marrow hemopoietic stem cells. Since it was not clear whether all the cells in our thymic low CD4 precursor

<table>
<thead>
<tr>
<th>Recipient organs</th>
<th>Cell injected (i.v.)</th>
<th>No. injected</th>
<th>Donor Ly 5.2+</th>
<th>Donor-type cells</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>Thy-1+</td>
</tr>
<tr>
<td>Thymus</td>
<td>B220−</td>
<td>30,000</td>
<td>1.5</td>
<td>97</td>
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<tr>
<td></td>
<td>B220+</td>
<td>4,500</td>
<td>0.1</td>
<td>−</td>
</tr>
<tr>
<td>Spleen and lymph nodes</td>
<td>B220−</td>
<td>30,000</td>
<td>9.1</td>
<td>68</td>
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The B220− and B220+ fractions of a low CD4 precursor population prepared without prior B220 depletion were sorted on the basis of staining analysis (Fig. 3). Both fractions were intravenously injected into irradiated mice differing at the Ly 5 locus. 4 wk later the recipient thymus, spleen, lymph nodes (pooled), and bone marrow were analyzed for donor-derived cells, as described in Fig. 5 and Materials and Methods. The level of reconstitution by the B220+ fraction was too low to determine the lineage distribution. The proportion of Mac-1+ and Gr-1+ cells among the Ly 5.2+ cells was within the background range (0−2.5%) for spleen and lymph nodes, and only marginally above it for bone marrow.
preparation were active as precursors, we used some additional markers in an attempt to subdivide the population. The markers were Sca-1 (Ly 6A/E), Sca-2, B220, and Rh-123 binding, all of which had been used previously for sub-dividing and characterizing bone marrow stem cells and precursor cells. Precursor activity was associated with the non-adherent, Sca-2−, B220−, and Rh-123hi cells, which represent the vast majority of cells in this preparation. We have now refined the isolation protocol to exclude any B220+ or adherent cells. The combination of this work and our previous study (7) indicates that the low CD4 precursor preparation consists almost entirely of CD4med, CD8−, CD3−, CD2−, B220−, IL-2Rα−, HSAmed, Thy-1−, class-I MHC−, class-II MHC−, Pgp-1(CD44)−, Rh-123−, Sca-1−, and Sca-2− cells, which are also negative for a series of non-T lineage markers. We have now confirmed the correlation of function with phenotype for 12 of these 14 markers. By these criteria the early precursor activity is associated with the phenotypically homogeneous majority (>95%) population, rather than with any minority subpopulation.

The fact that the thymic early precursor cells are Sca-1−, Sca-2− gives some insight into their relationship with bone marrow hemopoietic stem cells, which are Sca-1+, Sca-2− (5, 29). In a recent study by Spangrude and Scollay (29), purified bone marrow stem cells were injected into the thymus of irradiated recipients, and the phenotype of the donor-derived cells was determined. The only change detected 7 d after transfer was the acquisition of surface Sca-2. Only later did the progeny cells show the sequential expression of the markers characteristic of the later steps of thymocyte development. This result suggests that the cells in our low CD4 precursor population are the direct progeny of bone marrow hemopoietic stem cells, with the Sca-2 expression characterizing the early expansion phase in the thymus. The high level of Rh-123 staining, the appearance of the cells, their relatively high mitotic index, and our earlier cell-cycle analyses (7) are all consistent with this picture of an activated, expanding precursor population.

Developmental Potential of Thymic Low CD4 Precursors Compared with Bone Marrow Stem Cells. The second aspect of this study was to determine whether the thymic early precursor cells were multipotent like bone marrow stem cells, or restricted to T lymphocyte development, like the CD4−8− intrathymic precursor cells. The virtual lack of capacity to form erythroid or myeloid colonies in the spleen and in agar culture, in contrast to that of bone marrow stem cells, suggested that this thymic precursor population was restricted in developmental potential.

However, it could be argued that both these colony assays measure cells that are in a later stage of development than a true hemopoietic stem cell. Therefore, we tested the lineage commitment of the thymic low CD4 precursor after intravenous transfer, using the Ly 5 marker to trace the development of precursor cells into lymphoid and myeloid lineages. We made a direct comparison with an enriched population of bone marrow stem cells (Lin− BM), which enabled us to exclude the possibility that any activity measured in the thymic precursor population was due to a minor sub-population of contaminating multipotent stem cells. Because the developmental kinetics of the myeloid and lymphoid lineages are different, a full kinetic study of lineage reconstitution was undertaken.

It was clear that the number of Ly 5-bearing progeny cells obtained after intravenous transfer of the low CD4 precursors was much lower than that obtained from bone marrow stem cells. This finding may be due in part to the low CD4 precursors being poor competitors for developmental space with the host-type bone marrow stem cells, which were injected to rescue recipient animals from irradiation-induced death. However, it also suggests that the thymic precursors have a low potential for expansion, as compared with bone marrow stem cells. It was also notable that the thymic low CD4 precursors gave a transient reconstitution whereas the Lin− BM cells gave a more prolonged reconstitution. This all indicates that the low CD4 precursors have a relatively limited expansion potential compared with true self-renewing stem cell.

Despite the preference for thymic seeding, the low CD4 precursors did produce progeny in bone marrow, spleen, and lymph node. The striking finding was that not only were Thy-1+ T cell progeny found, but also B220+ B lineage cells. These B220+ progeny were surface Ig−, CD5−, small lymphoid cells, and so appeared to be normal B lymphocytes. However, no significant levels of Mac-1+ or Gr-1+ progeny were detected at any time point, an observation in agreement with the spleen colony assay. This observation indicates that the thymic low CD4 precursors had a developmental potential different from that of bone marrow hemopoietic stem cells. The B cell development from the thymic low CD4 precursor preparation was unlikely to be due to a low level of contamination with multipotent stem cells, since our Lin− BM preparation gave a quite different pattern of reconstitution. Nor is it likely to be due to contamination with the normal type of B220+ B-committed precursor cells (37), since all our B precursor activity was from the B220− majority population, and none was detected from a minor B220+ fraction when these were separately isolated.

A Lymphoid-committed Precursor? A restricted precursor cell common to the T and B lymphoid lineages is commonly assumed to exist in bone marrow, although direct evidence for this is meagre. The strongest evidence comes from long-term bone marrow cultures, where a restricted stem cell capable of generating both B and T lymphocytes has been detected (38–40). The results obtained in our study strongly suggest
the low CD4 precursor population from adult mouse thymus consists of lymphoid-committed precursors, which normally develop into both α/β and γ/δ T cells (7), but which retain a capacity to develop into B cells. Since the developmental fate of these thymic precursor cells is environmentally determined, the capacity to produce B cells may not be utilized within the thymus. Even when these precursors are injected intravenously, T cell development predominates, probably because of a bias to thymic seeding, but B cell development occurs from some of the precursors that seed bone marrow. This lack of absolute commitment to a particular lymphoid lineage agrees with our previous findings (7) that this population has TCR-β, TCR-γ, and IgH genes in germline state.

However, we cannot as yet eliminate the alternative possibility that our thymic low CD4 precursor population consists of a mixture of separate, already committed T and B precursors. The latter would have to be B220− B precursors, and the reason for their existence in the thymus would be obscure. Although our final low CD4 precursor population appears homogeneous on one- to four-color flow-cytometric analysis using 14 separate phenotypic markers, closely related but separate B and T precursors could have the same surface phenotype. Some form of clonal analysis will be required to demonstrate that a single precursor cell has both B and T developmental potential. This analysis will be difficult to obtain since an individual cell will tend to produce either B or T cells according to where it seeds, and because the expansion potential of these precursor cells is lower than that of bone marrow stem cells.

These studies raise some interesting questions about the stage at which development becomes lymphoid restricted. In contrast to recent results with human thymus (41), the earliest precursors we detected in the mouse thymus were already lymphoid restricted and distinguishable (by Sca-2 expression) from multipotent stem cells. However, we cannot tell whether they seeded the thymus in this state, or whether they became lymphoid restricted very soon after arrival, since most of the precursors we isolated must be expanded versions of the few cells originally seeding from bone marrow (42). We favor the view that the thymus is seeded by lymphoid-restricted cells, since if multipotent precursors are injected directly into the thymus, they produce substantial myeloid development, much more than normally detected within the thymus (29). In this context our current attempts to find an equivalent to our low CD4 precursor cell in bone marrow should provide some insights.

References


