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Characterization of a B Cell Progenitor Present in Neonatal Bone Marrow and Spleen But Not in Adult Bone Marrow and Spleen

Kim M. Moscatello, Kristen L. Biber, Deborah C. Dempsey, Robert Chervenak, and R. Michael Wolcott

The neonatal period marks an important time in mammalian immunologic development, yet it is often ignored in studies of lymphocyte development. We identified a cell population with the phenotype heat stable Ag (HSA)low lin CD43low that contained B cell progenitors at a high frequency in the neonatal bone marrow and spleen. Although cells with a similar phenotype can be identified in the bone marrow and spleen of adult animals, these populations showed a greatly reduced frequency of B cell progenitors. B lineage cells were detected after 7 days in culture at a frequency of 1:15 when HSAlow lin CD43low cells from neonatal bone marrow were cultured on stromal cells and IL-7 under limiting dilution conditions. Under similar conditions, the equivalent population in adult bone marrow had a frequency of B cell progenitors that was less than 1:2000. The expression of terminal deoxynucleotidyl transferase in freshly sorted neonatal HSAlow lin CD43low cells suggested that cells committed to the lymphocyte lineage were present in this population. These data suggested that the HSAlow lin CD43low population of cells represents a pool of B lineage precursors that may be responsible for filling the immune compartment early in neonatal life. The Journal of Immunology, 1998, 161: 5391–5398.

The ontogeny of the adult immune system begins early in gestation when pluripotent hematopoietic stem cells (PHSCs), which collect in the aorta-gonad-mesonephros, simultaneously seed the fetal liver, thymus, spleen, and bone marrow (1, 2). Consequently, B and T lymphocytes develop in a temporal manner that is believed to be dependent on the maturation of the hematopoietic microenvironment (2). More specifically, committed B lymphocytes are detected in the bone marrow at day 17 of gestation, and this is the site where active B lymphopoiesis occurs throughout the subsequent lifetime of the animal (2).

Much of what is known about B cell development has been focused on the fetal liver and the adult bone marrow, while B cell development during neonatal life has received less attention (3–6). The neonatal period represents a unique time in the life of the animal in that passive immunity from mother to infant is relatively short-lived and therefore the infant is left with the task of developing a mature and functional immune system. One hypothesis is that during neonatal life a period of rapid lymphocyte proliferation would be required until the immune system reaches a state of homeostasis, at which point rapid expansion of the immune system would no longer be needed. We are interested in learning the mechanisms that govern this phase of immune development.

Our studies of B cell ontogeny were focused on early lymphocyte progenitors because of their potential role in filling immune compartments early in life before steady state B lymphopoiesis is established in the animal. We have defined a unique cell population based on its differential expression of several lineage and functional markers known to be important in early hematopoiesis and B lymphopoiesis. This cell population was shown to be present only at a time during ontogeny when B lymphopoiesis and hematopoiesis are abundant, i.e., during neonatal development. This population of cells, which has the phenotype of heat stable antigen (HSA)low lin CD43low (lin, lineage mixture against Ter-119, Gr-1, Mac-1, and B220) and contributes to the pool of B lineage precursors in the spleen and bone marrow of neonatal mice but not in adult bone marrow or spleen after lymphopoiesis has achieved homeostasis.

Materials and Methods

Mice

Six- to 8-wk-old male and female C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME). C57BL/6J mice were bred, and the appearance of a vaginal plug was scored as day 0 of gestation. Postcoital female mice were individually caged and maintained on a high protein breeding diet (Harlan Teklad, Batonville, IL) during gestation.

Flow cytometry and cell sorting

Neonatal and adult mice were euthanized, and bone marrow and spleen were removed. Bone marrow was obtained by flushing the femurs and tibias with PBS supplemented with 1% BSA (PBS/BSA). Spleens were dispersed into single cell suspensions by grinding between the frosted ends of two glass slides. Cells were centrifuged at 200 × g for 10 min, resuspended in 1 ml of PBS/BSA, and counted with a hemacytometer. For flow cytometric analysis, staining reactions were conducted in 96-well U-bottom plates by resuspending 1 × 10^6 cells in 50 µl of the appropriately diluted Ab for 15 min on ice. Cells were washed with FACS buffer (PBS,
2% newborn calf serum, and 1 g/L sodium azide) and centrifuged at 200 × g for 3 min per wash. Samples were analyzed on an Epics Profile II and Elite work station software (Coulter, Hialeah, FL). Four-color analysis and cell sorting was done on a Vantage flow cytometer (Becton Dickinson, San Jose, CA), and analysis was accomplished with CellQuest software (Becton Dickinson, San Jose, CA). BFCs and debris were excluded on the basis of forward angle and 90° light scatter. Cell sorting and analysis were made available through the Louisiana State University Medical Center Core Facility for Flow Cytometry (Shreveport, LA).

For cell sorting, cells were stained in 15-mL conical centrifuge tubes using the appropriately diluted Ab at a concentration of 1 mL Ab/50 × 10⁶ cells. Cells were incubated on a shaking platform in a cold room at 4°C for 30 min. Cells were washed with sterile FACS buffer, without sodium azide, and centrifuged for 10 min at 200 × g. Stained cells were resuspended in a final volume of 50 × 10⁶ cells/mL. Deflected cells were collected in sterile FACS buffer and kept at 4°C.

Abs, unless otherwise indicated, were all purchased from PharMingen (San Diego, CA) and titrated before use to determine the optimal concentration for each Ab. Abs included: anti-CD45R (B220, clone RA3-6B2) conjugated to FITC, biotinylated anti-CD24 (HSA, clone M1/69), anti-CD43 (leukosialin, clone S7) conjugated to phycoerythrin, anti-Gr-1 (m-erythoid differentiation Ag, clone RB6-8C5) conjugated to FITC, and anti-Ter-119 (clone Ter-119), which was purchased unlabeled and was conjugated to FITC by dialyzing Ter-119 mAb against FITC (Sigma, St. Louis, MO) at a final concentration of 1 µg/mL FITC in 500 µl carbonate-bicarbonate buffer (0.2 M solution of anhydrous sodium carbonate and 0.2 M solution of sodium bicarbonate). Streptavidin-RED670 was purchased from Life Technologies (Gaithersburg, MD). Anti-Mac-1, clone M1/70 (7), and anti-FcRII, clone 2.4G2 (8), were produced at Louisiana State University Medical Center from clones purchased from American Type Culture Collection (Manassas, VA) and purified using protein G columns. Anti-Mac-1 was conjugated to FITC as described above for anti-Ter-119.

Colony assays in semisolid medium
Sorted cells were placed in Iscove’s modified Dulbecco’s medium (IMDM) (Sigma), with no pH indicator, and were serially diluted. Three hundred microliters of various concentrations of sorted cells were placed in 3 ml MethoCult M3434 (Stem Cell Technologies, Vancouver, Canada), vortexed, and incubated on ice for 10 min. Cells were then dispersed using a 5-mm syringe needle with a 23-gauge needle into two, 35 × 10 mm petri dishes, 1.1 ml volumes and placed into a larger petri dish (100 × 15 mm). A third petri dish filled with sterile buffer was placed into the larger petri dish and left uncovered to maintain a humidified environment. Cultures were incubated at 37°C in 6.5% CO2 for 12–18 days. Microscopic colonies were counted at various times over an 18-day period. MethoCult M3434 contains cytokine and growth factors IL-3, IL-6, stem cell factor, and erythropoietin and is capable of detecting CFU-erythroid, burst-forming unit-erythroid, CFU-megakaryocyte, CFU-granulocyte-macrophage, and CFU-granulocyte, erythroid, and monocyte/macrophage colonies. Each experiment was completed at least three times in duplicate.

In vitro culture and limiting dilution analysis
Sorted cells were cultured with irradiated (1800 rad via a 6-MeV linear accelerator) OP42 stromal cells (a generous gift from Dr. Paul Kincade, Oklahoma Research Foundation, Oklahoma City, Oklahoma) and 250 U/ml of recombinant human IL-7 (rHu IL-7; Genzyme, Cambridge, MA) in IMDM (supplemented with 10% FCS, glutamine, gentamicin, and 2-ME) under bulk culture or limiting dilution conditions. Supernatant from the 5358 IL-7 murine IL-7 producing cell line was also used as a source of IL-7 for the in vitro cultures (a generous gift from Drs. Antonius Rolink and Fritz Melchels, Basel Institute for Immunology, Basel, Switzerland). Cells cultured in bulk were harvested and analyzed at various time points for expression of B220 and HSA as described earlier. Bulk cultures were maintained weekly by aspirating 50% of the spent media and replacing it with fresh media or IL-7 only showed no colony formation. The cells cultured to determine the frequency of B cell progenitors in the purified cell populations. Cells were placed into culture on stromal cells with added IL-7 with 30 replicates of each culture. The cells were cultured for 1 wk and then scored for the presence of lymphoid colonies. The frequency of B cell progenitors in both the HSAlow lin -CD43low and HSAlow lin -CD43high populations was approximately 1 in 15 (Fig. 1, B and C, respectively). The identification of the lymphoid colonies as B lineage cells was confirmed by staining representative cultures with FITC-anti-B220 Abs, followed by flow cytometric analysis and treatment of positive cultures with LPS to induce the cells to differentiate and secrete IgM, which was detected by ELISA (data not shown). One possible source of the B cell progenitors in the two sorted cell populations is the presence of multipotent stem cells. Two methods were used to determine the presence of multipotent stem cells. The
first method was based on numerous reports (11–14) that detection of cobblestone area forming cells (CAFC), when bone marrow cells are cultured on a stromal layer, can be equated with the presence of pluripotent or omnipotent stem cells. Both cell populations were cultured under limiting dilution conditions for more than 4 wk, suggesting the presence of PHSC in this population of cells.

The second method used to detect multipotential stem cells in the sorted populations was culture in semisolid medium containing a mixture of cytokines that allow for the detection of hematopoietic progenitors. Various concentrations of HSA-low lin–CD43low/high cells were sorted from neonatal bone marrow and plated in MethoCult methylcellulose medium. The plates were cultured for 12–18 days at 37°C and scored for microscopic colonies. Nonlymphoid colonies were detected at a frequency of 1 in 75 within the HSA-low lin–CD34low population and at a frequency of 1 in 12 within the HSA-low lin–CD34high population (Table I). While the MethoCult system is capable of detecting monocyte/macrophage, granulocyte, and erythroid colonies, the HSA-low lin–CD34low population produced only colonies of the monocyte/macrophage lineage, whereas the HSA-low lin–CD34high population produced multipotent colonies consisting of monocyte/macrophages, granulocytes and erythroid cells and various combinations thereof. When whole bone marrow was used as a control, colonies of several different lineages were also detected. Together, these data suggest that the HSA-low lin–CD34high population contains primitive hematopoietic progenitors whereas the HSA-low lin–CD34low population contains cells that are developmentally more restricted. Because the HSA-low lin–CD34low population of cells appeared to be less primitive, we focused on this population as a candidate population for neonatal B cell progenitors.

The frequency of B cell progenitors in the HSA-low lin–CD34low population of neonatal spleen was examined as well. The HSA-low lin–CD34low population was sorted from the spleens of 2–3-wk-old neonatal mice and placed into culture under limiting dilution conditions on stromal cells and IL-7 as described above for the neonatal bone marrow. Pre- and postsort analyses are shown in Fig. 2A. The frequency of B cell progenitors in the HSA-low lin–CD34low population of neonatal spleen was calculated to be approximately 1 in 285 (Fig. 2B). Thus, while the B cell progenitor activity within the HSA-low lin–CD34low population is less frequent in the neonatal spleen than in neonatal bone marrow, the splenic HSA-low lin–CD34low population present in neonates is capable of B cell genesis.

Table I. Hematopoietic colony assays in semi-solid medium

<table>
<thead>
<tr>
<th></th>
<th>HSA-low lin–CD34low/high</th>
<th>Neutrophil Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD43low</td>
<td>CD43high</td>
</tr>
<tr>
<td>No. of cells seeded</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Mean no. of colonies</td>
<td>2 ± 0.8</td>
<td>12.6 ± 4.9</td>
</tr>
<tr>
<td>Frequency in bone marrow</td>
<td>1 in 75</td>
<td>1 in 12</td>
</tr>
<tr>
<td>Colony types</td>
<td>Monocyte/macrophage</td>
<td>Monocyte/macrophage,</td>
</tr>
</tbody>
</table>

a HSA-low lin–CD34low/high cells were sorted from neonatal bone marrow and cultured in MethoCult as described in Materials and Methods. Whole neonatal bone marrow was used as a control. Each experiment was performed in duplicate at least three times.

b Results represent the mean ± SD of colonies detected per dilution.

c Frequency was obtained by dividing the mean number of colonies by the number of cells seeded per well.

d No colonies were detected in cultures of sorted cells in which the cytokines were omitted.
In vitro differentiation and proliferation of HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cells

During differentiation of PHSCs along the B lineage pathway, the cells acquire B220 and up-regulate the expression of HSA (3, 15). Thus, the acquisition of HSA\textsuperscript{high} B220\textsuperscript{+} was used to assess the rate of differentiation of the sorted HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cells. The HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} population of cells was sorted from neonatal bone marrow and plated at 1 x 10\textsuperscript{4} cells/well in 12-well plates on irradiated stromal cells with IL-7. Cells were analyzed daily by flow cytometry for the change in expression of HSA and B220. After 1 day in culture, there was little differentiation into a HSA\textsuperscript{high} B220\textsuperscript{+} phenotype. However, by day 4 in culture, 13.1% of the cells had differentiated, and by day 6 of culture, 38.5% of the cells exhibited a differentiated phenotype (Fig. 3).

Four-color flow cytometric analyses were used to determine the extent of differentiation of the HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cells after 4 wk in culture. The Hardy system of B cell nomenclature was used to determine the extent of differentiation of the HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cells in culture (3). This system of B cell nomenclature is based on the differential expression of HSA, B220, CD43, BP-1, IgM, and IgD and is used to define B cell subpopulations from the least mature B cell (fraction A) to the most mature B cell (fraction F) (3). HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cells were sorted from neonatal bone marrow as described above and cultured in bulk on irradiated stromal cells with exogenous IL-7 for 4 wk. Cells were removed and labeled with Abs specific for HSA, B220, CD43, BP-1, IgM, and IgD and is used to define B cell subpopulations from the least mature B cell (fraction A) to the most mature B cell (fraction F). HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cells were sorted from neonatal bone marrow as described above and cultured in bulk on irradiated stromal cells with exogenous IL-7 for 4 wk. Cells were removed and labeled with Abs specific for HSA, B220, CD43, BP-1, IgM, and IgD. While cells within fraction A–D were detected, the majority of cells had the phenotype B220\textsuperscript{+}, CD43\textsuperscript{−}, IgM\textsuperscript{−}, IgD\textsuperscript{−}; fraction D contained B220\textsuperscript{−}, CD43\textsuperscript{−}, IgM\textsuperscript{−}, IgD\textsuperscript{−}; and fraction F contained B220\textsuperscript{−}, CD43\textsuperscript{−}, IgM\textsuperscript{−}, IgD\textsuperscript{−}.

The proliferative capacity of the HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cells in vitro was determined by performing daily cell counts. After 2 days in culture, the cell number did not substantially increase. However, over the next 4 days of the experiment the cell number increased approximately fourfold (Fig. 5). While the expansion of the HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cultures coincided with the time point at which the cells differentiated (Fig. 3), it is impossible to conclude whether the HSA\textsuperscript{low} lin\textsuperscript{−} CD43\textsuperscript{low} cells are proliferating or if...
proliferation is the result of a subsequent phenotype, such as fraction C.

**HSA low lin** \(^2\) CD43 low cells in adult bone marrow and spleen have a low frequency of B cell progenitors

Because the HSA low lin \(^2\) CD43 low cells from bone marrow and spleen contained a high frequency of B lymphocyte progenitors during neonatal life, we questioned whether a similar population from adult bone marrow had B cell progenitor activity as well. Adult bone marrow (Fig. 6A) and spleen (Fig. 6B) were collected, stained, sorted, and cultured under limiting dilution conditions as described above. HSA low lin \(^2\) CD43 low populations were detected in both the spleen and bone marrow of adult mice; however, when examined for lymphoid colonies in B cell limiting dilution assays, few if any lymphoid colonies were detected (frequency, 1:2000) even though the initial cell concentrations for the adult limiting dilution assays were three (spleen) and four (bone marrow) times the initial concentrations plated for the equivalent populations in neonatal mice (Table II).

**HSA low lin** \(^2\) CD43 low cells express TdT in neonatal and adult mice

The HSA low lin \(^2\) CD43 low cells from neonatal bone marrow were analyzed for the expression of TdT as an indication of lymphoid lineage commitment. TdT is a lymphocyte-specific protein whose expression is limited predominantly, if not exclusively, to B and T cells early in their developmental pathways (16–19). TdT expression by freshly sorted and 1-wk-old cultures of HSA low lin \(^2\) CD43 low cells was measured by Western blot analysis. Thymocytes served as a positive control and splenocytes as the negative control. As shown in Fig. 7, TdT was detected in whole cell lysates of thymus, cultured HSA low lin \(^2\) CD43 low cells, and freshly sorted HSA low lin \(^2\) CD43 low cells, but not in splenocytes. The expression of TdT by the HSA low lin \(^2\) CD43 low cells is consistent with the hypothesis that lymphocyte-committed precursors reside within this population.

TdT expression was also examined from the HSA low lin \(^2\) CD43 low population in adult bone marrow to determine whether the HSA low lin \(^2\) CD43 low cells from adult bone marrow have lost the capacity for B cell genesis via a suppressive mechanism in

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**Table II. Frequency of B cell progenitors from HSA low lin** \(^2\) CD43 low fraction of adult bone marrow and spleen**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell Dilutions</th>
<th>% Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA low lin (^2) CD43 low adult bone marrow</td>
<td>2,000</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>HSA low lin (^2) CD43 low adult spleen</td>
<td>1,500</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Whole adult bone marrow</td>
<td>10,000</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

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\(a\) HSA low lin \(^2\) CD43 low cells were sorted from adult bone marrow and spleen and cultured under limiting dilution conditions as described in Material and Methods. Whole, unseparated adult bone marrow was used as a positive control. Each experiment was completed at least three times and the results shown are a representative experiment.

\(b\) Cell dilutions varied between experiments and were dependent on the number of cells recovered from each cell sort.

\(c\) The percentage of negative cells for each dilution was determined by dividing the number of negative wells by the total number of wells and multiplying by 100.
adult animals or because the adult HSA<sub>low</sub> CD43<sub>low</sub> population is comprised of significantly fewer B lineage progenitors. TdT expression was analyzed in HSA<sub>low</sub> CD43<sub>low</sub> cells isolated from adult bone marrow as described above. Thymocytes and 1-wk-old cultures from neonatal HSA<sub>low</sub> CD43<sub>low</sub> cells served as positive controls, while NIH3T3 cells served as a negative control. As shown in Fig. 8, TdT was detected in HSA<sub>low</sub> CD43<sub>low</sub> cells isolated from adult bone marrow. TdT expression was not detected in NIH3T3 cells. These data suggest that the HSA<sub>low</sub> CD43<sub>low</sub> population from adult bone marrow still contains precursors of the B cell lineage, but at a much lower frequency than the equivalent population in neonatal mice.

**Discussion**

Here we describe a novel population of B lineage precursors that expresses low levels of HSA and CD43 and is negative for the lineage markers B220, Mac-1, Ter-119, and Gr-1. The cell surface proteins by which we have characterized the HSA<sub>low</sub> CD43<sub>low</sub> population are not unique to the B cell lineage. In fact, HSA and CD43 are expressed on a wide variety of lymphohematopoietic cells during at least some part of their development, including T cells, macrophages, erythroid cells, and hematopoietic stem cells (3, 20–24). However, it is the coordinate and temporal regulation of HSA and CD43 in the absence of other lineage marker expression that distinguishes the HSA<sub>low</sub> CD43<sub>low</sub> population of B cell progenitors from other precursors of the B cell lineage. Furthermore, this is the first report of a B cell population that is temporally regulated, because B cell progenitor activity is detected at a high frequency from the HSA<sub>low</sub> CD43<sub>low</sub> population in the bone marrow and spleen of neonatal mice and not in the bone marrow and spleen of adult animals. Not only was the frequency of B cell progenitors less in the adult HSA<sub>low</sub> CD43<sub>low</sub> population when compared with the neonatal HSA<sub>low</sub> CD43<sub>low</sub> population, but the total number of B cell progenitors was also considerably less in the adult population. In experiments testing as many as 3000 adult HSA<sub>low</sub> CD43<sub>low</sub> cells/well (data not shown), limiting dilution of 1 cell/well was never reached. Therefore, the frequency was <1:3000. Based on a frequency of <1:3000, the total number of B cell progenitors in the HSA<sub>low</sub> CD43<sub>low</sub> population sorted from the femurs and tibias of adult mice was <1.0 × 10<sup>5</sup> compared with >1.0 × 10<sup>6</sup> B cell progenitors sorted from the equivalent bones in neonatal mice. Thus, neonatal animals contain at least 100-fold more HSA<sub>low</sub> CD43<sub>low</sub> B cell progenitors than adult animals.

Differentiation of the HSA<sub>low</sub> CD43<sub>low</sub> cells to the B cell lineage was determined to characterize the subsequent phenotypes
of the HSA<sup>low</sup> CD43<sup>low</sup> cells after in vitro culture. The HSA<sup>low</sup> CD43<sup>low</sup> cells were determined to differentiate into cells expressing B220 and high levels of HSA within 4 days of culture on stromal cells with exogenously supplemented IL-7. Furthermore, the HSA<sup>low</sup> CD43<sup>low</sup> cells are capable of differentiating into fractions A–D when grown under conditions favoring B cell development. These data suggest that the HSA<sup>low</sup> CD43<sup>low</sup> cells developmentally precede fraction A cells as the HSA<sup>low</sup> CD43<sup>low</sup> cells give rise to fraction A cells in vitro. However, because the percentage of fraction A cells in culture was extremely low, it is possible that the HSA<sup>low</sup> CD43<sup>low</sup> cells parallel fraction A in the B cell developmental pathway.

Cell proliferation experiments revealed that cell division occurred from the HSA<sup>low</sup> CD43<sup>low</sup> population in vitro. It is impossible to extrapolate from these experiments whether the HSA<sup>low</sup> CD43<sup>low</sup> cells differentiate into B lineage precursors and then divide, possibly after having reached fraction C, or whether the HSA<sup>low</sup> CD43<sup>low</sup> cells undergo a few rounds of cell division and then differentiate along the B cell developmental pathway.

The multipotentiality of the HSA<sup>low</sup> CD43<sup>low</sup> cells was tested in vitro using hemopoietic colony assays. These studies showed that cells in the HSA<sup>low</sup> CD43<sup>low</sup> population of neonatal bone marrow not only had cells capable of development into B lineage cells, but also a low frequency of cells with the potential to develop into cells of the monocyte/macrophage lineage. Whether the HSA<sup>low</sup> CD43<sup>low</sup> cells represent a population of bi/multipotent progenitors capable of differentiation into multiple hematopoietic lineages or represent two populations each committed to a particular lineage cannot be resolved from these experiments. However, the HSA<sup>low</sup> CD43<sup>low</sup> population from neonatal bone marrow had at least a subpopulation of cells that expressed the lymphocyte-specific protein TdT. These data are consistent with the hypothesis that the HSA<sup>low</sup> CD43<sup>low</sup> cells are not multipotent for B cells and macrophages, but rather contain B220<sup>−</sup> precursors that are, at the very least, committed to the lymphoid lineage.

The HSA<sup>low</sup> CD43<sup>low</sup> population contains a high frequency of B cell progenitors, as demonstrated by limiting dilution analysis on stromal cells and IL-7. However, the fact that the HSA<sup>low</sup> CD43<sup>low</sup> population contributes to the generation of B cells does not constitute the uniqueness of this population, as several populations of primitive B cell progenitors have been previously described. For example, Cumano et al. characterized a bipotential precursor of B cells and macrophages from day 12 fetal livers that are similar to the HSA<sup>low</sup> CD43<sup>low</sup> B cell precursors that we have described in that the bipotential cells are negative for the expression of B220 and Mac-1 (25). Other similarities between the B cell/macrophage precursor and the HSA<sup>low</sup> CD43<sup>low</sup> population include the fact that we have isolated and cloned HSA<sup>low</sup> CD43<sup>low</sup> cells from the fetal liver (data not shown), and it appears that they have the potential to develop into both B cells and macrophages. However, the B cell/macrophage precursor has never been convincingly demonstrated in the bone marrow of adult or young mice. Furthermore, the frequency of B cell progenitors within the bipotential cell population was increased when isolated based on the expression of the cell surface protein AA4.1 (26), whereas the HSA<sup>low</sup> CD43<sup>low</sup> population was not increased in frequency by the inclusion of AA4.1 in the sorting strategy (data not shown). Thus, we initially entertained the possibility that the bipotential B cell/macrophage precursors and the HSA<sup>low</sup> CD43<sup>low</sup> cells were equivalent populations. However, based on the data presented here we believe that they represent two separate populations of cells.

Li et al. have described, as of yet, the earliest B lineage precursor in the adult bone marrow (27, 28). This population, designated A<sub>1</sub>, is a subdivision of fraction A cells initially described by Hardy several years earlier (3, 27, 29). The A<sub>1</sub> subpopulation shares some common features of both the bipotential B cell/macrophage precursors and the HSA<sup>low</sup> CD43<sup>low</sup> population, yet bears some distinctive features as well. The A<sub>1</sub> subpopulation and the HSA<sup>low</sup> CD43<sup>low</sup> cells are distinguishable in the expression of B220 and HSA, as A<sub>1</sub> is positive for the common leukocyte Ag B220 and lacks the expression of the HSA glycoprotein, whereas the HSA<sup>low</sup> CD43<sup>low</sup> cells have not yet acquired B220 on the cell surface and have begun to express low levels of HSA (27). Regardless of the similarities between the A<sub>1</sub> subpopulation of B cell progenitors and the HSA<sup>low</sup> CD43<sup>low</sup> population, the paramount difference is in the fact that we have not been able to isolate HSA<sup>low</sup> CD43<sup>low</sup> cells with B cell potential from the spleen or bone marrow of adult animals in amounts equivalent to neonatal bone marrow, the implications of which are discussed below.

Recently, Kondo et al. have published a report describing a cell population with the phenotype lin<sup>−</sup> IL-7R<sup>−</sup> Thy-1<sup>−</sup> Sca-1<sup>−</sup> c-kit<sup>−</sup> that is restricted to the lymphoid lineage (T, B, and NK cells) (30). This common lymphoid progenitor (CLP) also has the phenotype of HSA<sup>low</sup> CD43<sup>low</sup>, which is similar to the HSA<sup>low</sup> CD43<sup>low</sup> population described in this report. The status of the IL-7R on the surface of the HSA<sup>low</sup> CD43<sup>low</sup> cells is currently unknown because the IL-7R Ab was unavailable at the time these studies were completed. However, the HSA<sup>low</sup> CD43<sup>low</sup> population is c-kit<sup>−</sup> and Thy-1<sup>−</sup>, and a fraction of HSA<sup>low</sup> CD43<sup>low</sup> cells were positive for Sca-1 (data not shown). Furthermore, in preliminary studies (K.M.M. et al., manuscript in preparation) we have found that the HSA<sup>low</sup> CD43<sup>low</sup> population from neonates, but not from adults, was capable of reconstituting the B and T cell compartments of Rag1<sup>−/−</sup> mice, suggesting that CLPs may exist within this population in neonatal mice but not in the analogous population from adults. Taken together, the HSA<sup>low</sup> CD43<sup>low</sup> population and the CLP population described by Kondo et al. appear to be similar in phenotype and activity. However, the HSA<sup>low</sup> CD43<sup>low</sup> population we have characterized contains monocyte progenitors that have been demonstrated in vitro, whereas monocyte progenitor activity has not been documented in the CLP population. Finally, in comparing the CLP from Kondo’s group to the HSA<sup>low</sup> CD43<sup>low</sup> cells described in this paper, the CLP were isolated from adult murine bone marrow (between 4–10 wk of age), whereas the HSA<sup>low</sup> CD43<sup>low</sup> population appears to be active primarily in the neonatal period. Because the precise time that the HSA<sup>low</sup> CD43<sup>low</sup> population becomes diminished in its capacity to generate B cells is currently under investigation, it is not known whether the CLP and the HSA<sup>low</sup> CD43<sup>low</sup> population from neonatal mice are equivalent populations.

Although the HSA<sup>low</sup> CD43<sup>low</sup> population of cells appears to be an early intermediate in the B cell developmental pathway, it is interesting that the HSA<sup>low</sup> CD43<sup>low</sup> B lineage precursors were detected at a high frequency in the spleen and bone marrow of neonatal mice but not in the spleen and bone marrow of adult mice. However, the HSA<sup>low</sup> CD43<sup>low</sup> cells from adult bone marrow express the lymphocyte-specific protein TdT, suggesting that the adult HSA<sup>low</sup> CD43<sup>low</sup> population still contains cells with B lineage potential but at a low frequency. This is the first demonstration of a unique developmental intermediate that may be involved in the genesis of the immune system in neonatal mice. We hypothesize that the HSA<sup>low</sup> CD43<sup>low</sup> cells are present at
an important point during ontogeny when the lymphocyte compartment is likely in a state of rapid expansion. The HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} cells would serve the function of expanding the B lymphocyte compartment of the immune system until homeostasis is achieved. Once the lymphocyte compartment has reached steady state levels, the HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} population would no longer be required and would thus decrease, but remain in the bone marrow and/or spleen at extremely low frequencies. In recent experiments (K.M.M. et al., manuscript in preparation), we observed that irradiation of adult animals resulted in an increase of B cell progenitor activity in the HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} population of cells isolated from adult bone marrow. This is consistent with the hypothesis that the HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} cells are up-regulated during periods where rapid lymphocyte expansion is necessary.

A second explanation for the “disappearance” of the HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} cells in adult mice concerns the effects of age on the immune system. The effects of aging on B cell development (28, 31–33) and hematopoiesis (34–36) have been well documented. And, while the effects of aging on the capacity of hematopoietic stem cells to self-renew is somewhat controversial, there are several lines of evidence to suggest that the bone marrow in aged mice is diminished in its capacity to generate B cells (28, 31, 32). Recent reports have further demonstrated that the decline of B lymphopoiesis associated with aging reflects a diminished ability of the pre-B cell fraction to proliferate (31). Furthermore, the defect in the proliferative capacity of pre-B cells was determined to be related to the impaired ability of pro-B cells to respond to IL-7 (28). The HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} cells described in this report represent a point in B cell development that is before the pro-B cell stage by several different criteria. Because there was no decline in the number of pro-B cells reported by Stephan et al., which might be indicative of a problem preceding the pro-B cell stage, we do not believe that the ontologic specificity observed in the HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} population is due to the effects of aging on the immune system.

A third hypothesis for the failure of the HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} cells from adult bone marrow to produce B cell progenitors is that the adult cells have different growth factor requirements for proliferation and differentiation. This hypothesis seems unlikely because the culture system that was used is capable of supporting PHSCs.

In summary, we have characterized a population of HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} cells from neonatal bone marrow and spleen that contains a high frequency of B lineage progenitors. The most striking observation made is that the HSA\textsuperscript{low}lin\textsuperscript{−}CD43\textsuperscript{low} population is capable of yielding B lineage cells when isolated from neonatal mice but not when isolated from adults. While much of B cell development and lymphocyte development is, in general, focused on the adult murine bone marrow and the fetal liver, we have demonstrated that the bone marrow from neonatal mice is not analogous to the bone marrow from adult mice and thus the developmental events in one do not necessarily parallel the other.

References