Primitive Hematopoietic Cells in Murine Bone Marrow Express the CD34 Antigen

By Franck Morel, Stephen J. Szilvassy, Marilyn Travis, Benjamin Chen, and Anne Galy

The CD34 antigen is expressed on most, if not all, human hematopoietic stem cells (HSCs) and hematopoietic progenitor cells, and its use for the enrichment of HSCs with repopulating potential is well established. However, despite homology between human and murine CD34, its expression on subsets of primitive murine hematopoietic cells has not been examined in full detail. To address this issue, we used a novel monoclonal antibody against murine CD34 (RAM34) to fractionate bone marrow (BM) cells that were then assayed in vitro and in vivo with respect to differing functional properties. A total of 4% to 17% of murine BM cells expressed CD34 at intermediate to high levels, representing a marked improvement over the resolution obtained with previously described polyclonal anti-CD34 antibodies. Sixty percent of CD34+ BM cells lacked lineage (Lin) markers expressed on mature lymphoid or myeloid cells. Eighty-five percent of Sca-1-Thy-1-Lin- cells that are highly enriched in HSCs expressed intermediate, but not high, levels of CD34. The remainder of these phenotypically defined stem cells were CD34+. In vitro colony-forming cells, day-8 and -12 spleen colony-forming units (CFU-S), primitive progenitors able to differentiate into B lymphocytes in vitro or into T lymphocytes in SCID mice, and stem cells with radioprotective and competitive long-term repopulating activity were all markedly enriched in the CD34+ fraction after single-parameter cell sorting. In contrast, CD34- BM cells were depleted of such activities at the cell doses tested and were capable of only short-term B-cell production in vitro. The results indicate that a significant proportion of murine HSCs and multilineage progenitor cells express detectable levels of CD34, and that the RAM34 monoclonal antibody is a useful tool to subset primitive murine hematopoietic cells. These findings should facilitate more direct comparisons of the biology of CD34+ murine and human stem and progenitor cells.

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populations of BM cells. Consequently, unlike in humans,29 it has been more difficult to assess the functional properties of murine cells expressing different amounts of CD34. Furthermore, the lymphoid differentiation potential and long-term repopulating potential of CD34-selected murine BM cells has not yet been examined in detail.

To address these issues, we examined the expression of CD34 on murine BM cells using a recently available monoclonal antibody (MoAb), RAM34, with superior staining characteristics.30 Clonogenic hematopoietic cells were characterized using a variety of functional in vitro and in vivo assays including competitive long-term repopulation. We show that in vitro colony-forming cells (CFCs), CFU-S, competitive repopulating units (CRU), and progenitors able to differentiate into B lymphocytes in vitro or into T lymphocytes in SCID mice are highly enriched in the fraction of BM cells expressing intermediate to high levels of CD34.

MATERIALS AND METHODS

Animals. Four- to 6-week-old (C57BL/Ka.AKR/J)Sys-Ppcre- Thy-1 mice (B.A1; Thy-1.1, Ly-5.2) were used as BM cell donors. Six- to 8-week-old Ly-5 congenic (C57BL/6.JSlJ)/Sys-Ppcre-Thy-1 mice (B6.SJL; Thy-1.2, Ly-5.1) were used as recipients for competitive repopulation experiments. All mice were bred and maintained on sterilized food and acidified water at the SyStemix Inc animal facility.

Preparation of BM cell suspensions. Femoral BM cells were obtained from B.A1 mice and cell suspensions prepared in phosphate-buffered saline containing 0.2% bovine serum albumin (PBS-BSA) by repeated flushing of the BM through a 21-gauge needle, followed by filtration through a 20-μm nylon mesh. Cells were washed twice in PBS-BSA, layered on Isopaque medium (Robbins Scientific, Sunnyvale, CA), and centrifuged at 1,000g for 20 minutes at room temperature. Cells at the interface were collected and washed twice in PBS-BSA, counted, and resuspended at 10^7/mL in PBS-BSA containing 1 mg/mL heat-inactivated human γ-globulin (Gambro, Milan, Italy) and 10 μg/mL rat antimony mouse CD16/ CD32 to block nonspecific antibody binding (omitted in experiments to identify Sca-1-Thy-1^+Lin^- stem cells).

Immunolabeling and flow cytometric sorting. The MoAbs (all from Pharmingen, San Diego, CA, except ALI4A2) used for flow cytometric analysis and sorting are shown in Table 1. BM cells were incubated with biotinylated rat anti-CD34 MoAb (RAM34) for 30 minutes on ice, washed twice in PBS-BSA, and stained with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Caltag, San Francisco, CA) for 30 minutes. Control cells were stained with biotinylated rat-IgG₂ (clone R35-95; Pharmingen) and streptavidin-FITC. In some experiments, BM cells were first stained for 30 minutes with a cocktail of unlabelled lineage antibodies specific for CD3, CD5, CD8, Gr-1, Mac-1, and B220 and then were washed and stained with Texas red-conjugated goat antirat IgG (Southern Biotechnology Associates Inc, Birmingham, AL). The cells were washed, and unbound antirat paratopes were blocked by incubation in 100 μg/mL of purified rat IgG (I-4131; Sigma Chemical Co, St Louis, MO). Cells labeled with lineage antibodies were then incubated with biotinylated anti-CD34, phycocerythrin (PE)-conjugated anti-Sca-1, and Thy-1.1-FITC for 30 minutes and then were washed and stained with allophycocyanin (APC)-conjugated streptavidin. Labeled cells were finally washed twice in PBS-BSA and resuspended in PBS-BSA containing 10 μg/mL propidium iodide (Boehringer Mannheim, Indianapolis, IN). Viable "lymphoblastoid" cells were defined by low-to-intermediate forward (FSC) and side (SSC) light-scattering properties, and exclusion of propidium iodide on a
dual-laser FACStar Plus or Vantage cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were collected in 24-well plates into RPMI 1640 medium containing 10% fetal calf serum (FCS) and were counted and reanalyzed for purity (>90%) in every experiment.

In vitro colony assays. BM cells (5 × 10^5 to 5 × 10^6/dish) were plated in 35-mm petri dishes (Nunc, Naperville, IL) in 1.1 mL of Methocult M3330 (StemCell Technologies Inc, Vancouver, Canada) supplemented with 10 ng/mL recombinant murine interleukin-3 (IL-3), IL-6, granulocyte/macrophage colony-stimulating factor, 100 ng/mL murine Steel factor (SLF), and 2 U/mL recombinant human erythropoietin (all from Genzyme, Cambridge, MA). Duplicate cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and colonies of greater than 50 cells were counted after 12 days. These culture conditions allowed the growth of BFU-E, CFU-GM, and multilineage colony-forming units (CFU-GEMM).

CFU-S assays. BM cells (0.5 to 2.5 × 10^6/mouse) were injected via the lateral tail vein into groups of 4 lethally irradiated C57Bl/Ka recipient mice (10.5 Gy administered in 2 equal doses ≥3 hours apart using a 250Cs irradiator [JL Shepherd and Associates, San Fernando, CA]). Eight or twelve days later, mice were killed, and spleens were removed and fixed in Tellyesniczky's solution for macroscopic spleen colony counts.31

B-cell cultures. Sorted cells were cultured on an adherent monolayer of murine Sys-1 stromal cells. Sys-1 is a subclone of AC6 stromal cells,27 and supports the proliferation and limited differentiation of human CD34^"Thy-1^+Lin^-" and murine Thy-1^+Sca-1^-H-2K^- stem cells.33,34 Sys-1 stromal cells were plated in 96-well flat-bottom tissue culture plates (10^5/well) in 100 μL of medium consisting of 50% Iscove's modified Dulbecco's medium and 50% RPMI-1640 (GIBCO-BRL, Gaithersburg, MD) with 5% FCS, 0.05 mmol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 4 mmol/L glutamine (URH Biosciences, Lenexa, KS). Approximately 7 days later, 10^5 sorted cells were added to each well in a volume of 100 μL. Cultures were incubated at 37°C and fed biweekly by demidepletion. Five to 10 wells were harvested weekly, cells were counted, and B-lineage cells were identified by staining with anti-B220-FITC and anti-CD34-PE, or anti-B220-PE and anti-sIgM-FITC MoAbs using a FACScan instrument (Becton Dickinson).

Table 1. MoAbs

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT3.1</td>
<td>CD3</td>
</tr>
<tr>
<td>GK1.5 or RM4.5</td>
<td>CD4</td>
</tr>
<tr>
<td>53-3.131</td>
<td>CD5</td>
</tr>
<tr>
<td>53-6.72</td>
<td>CD8</td>
</tr>
<tr>
<td>M1/70</td>
<td>CD11b (Mac-1)</td>
</tr>
<tr>
<td>2.402</td>
<td>CD16/32</td>
</tr>
<tr>
<td>RAM34</td>
<td>CD34</td>
</tr>
<tr>
<td>57</td>
<td>CD43</td>
</tr>
<tr>
<td>14.8</td>
<td>CD49RA</td>
</tr>
<tr>
<td>RA3-6B2</td>
<td>B20</td>
</tr>
<tr>
<td>RB6-RC5</td>
<td>Gr-1</td>
</tr>
<tr>
<td>HIS51 or 19X5</td>
<td>Thy-1.1</td>
</tr>
<tr>
<td>E13-161.7</td>
<td>Sca-1</td>
</tr>
<tr>
<td>II/41</td>
<td>surface IgM</td>
</tr>
<tr>
<td>AF6-88.5</td>
<td>H-2K&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>288</td>
<td>c-kit</td>
</tr>
<tr>
<td>H57-597</td>
<td>TCR-αβ</td>
</tr>
<tr>
<td>ALI4A2</td>
<td>Ly-5.2</td>
</tr>
</tbody>
</table>

T-cell differentiation assay. Fetal thymic lobes were obtained from CB.17 ( Thy-1.2<sup>+</sup>) scid/scid (SCID) fetuses on day 14 of gesta-
tion. Thymic lobes were placed in a Terasaki plate together with 10^5 "test" cells (Thy-1.1) per 20 mL of medium consisting of 50% Iscove’s modified Dulbecco’s medium, 50% RPMI 1640, 10% FCS, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium (Sigma), MEM vitamins (GIBCO-BRL), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.05 mmol/L 2-mercaptoethanol, and 4 mmol/L glutamine. Seeding of the thymic lobes was performed using the "hanging drop" method by inversion of the plate and incubation for 48 hours at 37°C. After seeding, thymic lobes were implanted under the kidney capsule of anesthetized 6- to 8-week-old (Thy-1.2^+) SCID mice. After 3, 5, or 7 weeks, recipients were killed, and the thymic lobes were retrieved and homogenized in PBS-BSA containing 0.02% NaN_3 using a microtissue grinder (Wheaton, Millville, NJ). Cells were washed and counted, and donor-derived T lymphocytes were identified by staining with biotinylated anti-Thy-1.1, anti-CD3-FITC, anti-CD4-APC, anti-CD8-PE, and anti-αβ-cell receptor (anti-αTCRαβ)-FITC MoAbs. Statistical significance was determined using the paired Student’s t-test.

Radioprotection and competitive repopulation of lethally irradiated mice. Ly-5.1 mice were irradiated as for CFU-S assays and intravenously injected with 0.2 mL of medium containing no cells or 2 x 10^5 unfractionated or CD34-selected Ly-5.2^+ BM cells, together with 2 x 10^5 twice serially transplanted 'compromised' Ly-5.1^+ BM cells. Compromised BM cells contain normal numbers of relatively mature progenitors and are radioprotective. However, CRU have been reduced to a frequency of less than 1 per 250,000 cells, thereby minimizing competition against "test" HSCs during the later stages of engraftment. Recipients were maintained on drinking water containing 0.55 mg/mL neomycin sulfate and 840 mg/L streptomycin A (Sigma), MEM vitamins (GIBCO-BRL), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.05 mmol/L 2-mercaptoethanol, and 4 mmol/L glutamine. Seeding of the thymic lobes was performed using the "hanging drop" method by inversion of the plate and incubation under the kidney capsule of anesthetized 6- to 8-week-old (Thy-1.2^+) SCID mice. After 3, 5, or 7 weeks, recipients were killed, and the thymic lobes were retrieved and homogenized in PBS-BSA containing 0.02% NaN_3 using a microtissue grinder (Wheaton, Millville, NJ). Cells were washed and counted, and donor-derived T lymphocytes were identified by staining with biotinylated anti-Thy-1.1, anti-CD3-FITC, anti-CD4-APC, anti-CD8-PE, and anti-αβ-cell receptor (anti-αTCRαβ)-FITC MoAbs. Statistical significance was determined using the paired Student’s t-test.

RESULTS

Expression of CD34 on murine BM cells. A rat MoAb, RAM34, defined by its reactivity to cells transfected with a cDNA encoding murine CD34, was used to characterize the expression of the CD34 antigen on BM cells from B.A1 mice. In B.A1 BM, 5% ± 2% (range, 4% to 17%) of cells expressed the CD34 antigen. The majority of CD34^+ cells expressed intermediate levels of CD34 antigen, although a small population of CD34-bright cells was consistently detected (Fig 1). Most CD34^+ cells showed a blastoid morphology with intermediate FSC and SSC. To correlate the expression of CD34 with antigens previously described on HSCs and/or progenitors, and to analyze its coexpression with lineage-associated antigens, multicolor flow cytometric analyses were performed. A proportion of CD34^+ cells express antigens characteristic of B lymphocytes (B220 [21%]), T lymphocytes (Thy-1 [6%], CD4 [17%], or CD8 [9%]), and granulocytes and monocytes (Gr-1 [16%], Mac-1 [42%]). Interestingly, a significant percentage of CD34^+ cells also expressed CD45RA (26%), Sca-1 (11%), CD43 (59%), H-2K (88%), or c-kit (16%) antigens, which have been described on different subsets of HSCs or progenitors (Fig 1). Approximately 60% of CD34^+ cells did not express or expressed only a low level of the markers included in our Lin panel (CD3, CD5, CD8, Gr-1, Mac-1, and B220; see Fig 2B). To directly assess the expression of CD34 on a phenotypically defined subset of BM cells enriched in HSCs, we performed 5-color analysis of Sca-1^+Thy-1^+Lin^- BM cells gated as shown in Figs 2A and B. Sca-1^+Thy-1^+Lin^- cells are 1,000-fold enriched in hematopoietic cells able to confer radioprotection, and form d.12 spleen colonies with near unit efficiency. The majority (85% ± 15%) of Sca-1^+Thy-1^+Lin^- cells were CD34^+ (Fig 2C). However, the presence of the remaining ±15% of Sca-1^+Thy-1^+Lin^- cells in the CD34^+ fraction suggests that some murine stem cells and/or progenitors may not express the CD34 antigen. Interestingly, cells expressing the highest levels of CD34 were predominantly Sca-1^- and Thy-1^- (Figs 2D and E). Overall, only ±1.5% of CD34^+ cells were Sca-1^-Thy-1^-Lin^-^, suggesting the presence of more mature hematopoietic cells in the CD34^- BM fraction.

The majority of CFCs and CFU-S express CD34. To examine the presence of CD34 on relatively mature hematopoietic progenitors, BM cells were separated into CD34^+ and CD34^- fractions and assayed for CFC and CFU-S activity. Although staining with this anti-CD34 MoAb did not yield discrete positive and negative subpopulations (Fig 1), reanalysis of sorted CD34^+ and CD34^- cells indicated a purity of 98% ± 2% (n = 14). Table 2 shows that BFU-E, CFU-GM and CFU-GEMM were enriched 10-fold, eightfold, and sixfold, respectively, in the CD34^- fraction as compared with unseparated B.A1 BM. Day-8 and d.12 CFU-S were also enriched ±4.5-fold among CD34^- BM cells. In contrast, CFCs and CFU-S were absent or severely depleted in the CD34^- fraction. The low level of CFU-GM and CFU-S activity may reflect the few (±2%) contaminating CD34^+ cells in the CD34^- population. Alternatively, the previous finding that ±15% of Sca-1^+Thy-1^+Lin^-^ cells are CD34^- is consistent with this observation and suggests that some CFU-S and/or CFU-GM may not express CD34. The overall recovery of CFCs and CFU-S in the CD34^- population varied from 73% to 100% (Table 2).

B-lymphoid differentiation potential of CD34^- and CD34^- BM cells. To determine the capacity of CD34^- and CD34^- BM cells to differentiate into B lymphocytes in vitro, cells were cultured without cytokines on SyS-I stromal cells that express IL-7 and SLF, and which have previously been shown to support B lymphopoiesis. Cultures initiated with 10^5 CD34^+ cells underwent significant proliferation, resulting in a maximum 153-fold ± 21-fold increase in nucleated cells after 7 days (Fig 3A). After 3 weeks, total cells were increased ±90-fold over the number present on day 0. CD34^- cells expanded only 34-fold ± 2-fold after 2 weeks and were almost completely exhausted after 3 weeks (Fig 3A). To analyze these cells in more detail, we examined their expression of cell surface markers characteristic of pro-B (B220^+CD43^-slgM^-), pre-B (B220^+CD43^-slgM^-), and
mature B cells (B220⁺CD43⁻slgM⁺).

Mature B cells could be detected at as early as 4 days in cultures initiated with CD34⁻ cells (data not shown). After 7 days, ≈30% of the CD34⁻ cells had differentiated into slgM⁺ B lymphocytes (8,500 ± 1,300 per culture), but these died rapidly and were almost undetectable after 3 weeks (Fig 3D). Primitive pro-B or pre-B progenitors represented 66% (18,900 ± 3,000 per culture) and 9% (2,600 ± 100 per culture), respectively, of the cells in 7-day cultures of CD34⁻ BM (Fig 3B). Pro-B–cell numbers then decreased concomitantly with a transient increase in pre-B cells (Fig 3C), and both compartments were exhausted by day 21. Mature B cells could not be detected in the cultures initiated with 10⁶ CD34⁺ BM cells BM until day 7 (Fig 3D). Thereafter, B-cell numbers increased sharply to 23,000 ± 2,000 per culture, or 26% of nucleated cells, on day 21. Pro-B and pre-B progenitors were also abundant at all times up to day 21, representing a maximum of 90% of the cells generated in cultures of CD34⁺ BM on day 14 (Figs 3B and C). Therefore, CD34⁺ BM cells appeared to take longer to differentiate and produced significantly more mature B cells than did CD34⁻ cells in this stroma-dependent culture system.

T-lymphoid differentiation potential of CD34⁺ and CD34⁻ BM cells. The T-cell differentiation capacity of the CD34 fractions was analyzed in a thymic reconstitution assay in which day-14 fetal SCID mouse thymi were seeded with 10⁴ sorted or unfractionated BM cells in vitro and then transplanted under the kidney capsule of SCID recipients. Donor T cells were distinguished from SCID host cells by their expression of the Thy-1.1 allele. Analyses of the cells from representative thymi are shown in Fig 4. No mature T cells (0.2% ± 0.2%) were detected in 11 control thymi cultured with medium alone (5 experiments). CD34⁺ BM cells generated a very low number of donor-derived cells (0.9% ± 0.4% in 23 thymi from 8 experiments), but no significant number of immature or mature T cells expressing CD3, CD4, CD8,
and TCRαβ could be detected (**P** = .44). In contrast, thymi reconstituted with either 10^7 unfractionated or CD34^+ BM cells contained a large number of cells after 7 weeks, of which 22% ± 9% (18 thymi, 6 experiments; **P** < .05) and 51% ± 9% (24 thymi, 8 experiments; **P** < .01), respectively, were of donor (Thy-1.1^+^) origin. Approximately 75% to 80% of the cells in the thymi seeded with CD34^+^ cells expressed CD3 or TCRαβ, and mature CD4^+^ and CD8^+^ T lymphocytes were abundant. Furthermore, CD34^+^ BM cells generated threefold to sevenfold more mature (CD3^+, TCRαβ^+, CD4^+, or CD8^+) T cells that were more long-lived than those produced in thymi reconstituted by an equivalent number of unfractionated BM cells (Fig 4). T cells recovered from thymi reconstituted with unfractionated or CD34^+, but not CD34^−^, BM cells were also normally responsive to phytohemagglutinin and IL-2 in vitro (data not shown). These results indicate that murine T-lymphoid progenitors with in vivo proliferative potential are highly enriched among CD34^+^ BM cells.

Radioprotective and competitive long-term repopulating potential of CD34^+^ and CD34^−^ BM cells. The ability of
CD34-selected subpopulations to protect mice from the lethal effects of radiation-induced BM aplasia was analyzed by injecting irradiated mice with 10⁶ CD34⁻ or CD34⁺ BM cells. As shown in Fig 5, all 12 mice transplanted with CD34⁻ BM cells died by day 21 after irradiation and showed a survival curve that was indistinguishable from that of untransplanted controls. In contrast, 11 of 14 (79%) lethally irradiated mice injected with 10⁶ CD34⁺ BM cells survived at least 45 days. These results indicate that radioprotective cells are highly enriched among BM cells that express CD34.

To evaluate the expression of CD34 antigen on more primitive stem cells able to regenerate and maintain both lymphoid and myeloid lineages for several months after transplantation, we used a rigorous competitive repopulation assay.³⁵ Ly-5.2⁺ BM cells were fractionated into CD34⁻ and CD34⁺ subpopulations and injected at limiting dilution (2 to 50 × 10³ cells/mouse) into lethally irradiated Ly-5.1 congenic recipients together with 2 × 10⁵ "compromised" Ly-5.1⁺ BM cells. Compromised BM cells contain normal numbers of relatively mature progenitors and are radioprotective.³⁵,³⁶ However, CRU have been reduced to a frequency of less than 1 per 250,000 cells, thereby minimizing competition against "test" HSCs during the later stages of engraftment.³⁷ Four months after transplantation, the proportion of mice showing ≥5% donor-derived (Ly-5.2⁺) cells among B- and T-lymphoid and myeloid lineages was used to calculate the

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**Table 2. Frequency of in Vitro CFCs and CFU-S in CD34⁻ and CD34⁺ BM**

<table>
<thead>
<tr>
<th>Unseparated BM</th>
<th>CD34⁻ BM</th>
<th>CD34⁺ BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (per 5 × 10⁴)</td>
<td>Frequency (per 5 × 10⁴)</td>
<td>Enrichment Factor</td>
</tr>
<tr>
<td>BFU-E</td>
<td>33 ± 6</td>
<td>0</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>184 ± 19</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>3 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>d.8 CFU-S</td>
<td>74 ± 16</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>d.12 CFU-S</td>
<td>61 ± 16</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

* Values are shown as the mean ± SEM measured in three independent experiments.
* BM cells were ficolled but otherwise unselected before analysis.
† Recovery values were calculated by multiplying the enrichment factor by the percentage of ficolled cells represented by the corresponding CD34-selected population for each experiment.
‡ Spleen colony counts not corrected for seeding efficiency.
Thymus reconstituted with Medium BM CD34- CD34+

CD3

TCR alpha-beta

Thy-1.1

Gated on CD3+ cells

CD3

CD4

CD8

Fig 4. T-lymphoid differentiation capacity of CD34- and CD34+ BM cells. Thy-1.2+ day-14 SCID fetal thymic lobes were reconstituted with medium or 10^6 unfractionated (BM), CD34- or CD34+ cells isolated from B.A1 (Thy-1.1) mice and were then transplanted into Thy-1.2+ SCID mice. Shown are the expression of CD3, TCRαβ, Thy-1.1, CD4, and CD8 on thymocytes recovered 7 weeks after transplantation, in one representative experiment of eight. The percentage of total cells expressing each antigen is indicated in each panel.
frequency of CRU in the original sorted population. All 18 mice cotransplanted with $10^4$ or $5 \times 10^4$ CD34$^-$ (and $2 \times 10^5$ compromised) BM cells contained only host-type PB leukocytes (Table 3). In contrast, 3 of 8, 8 of 10, and 15 of 15 mice transplanted with $2 \times 10^3$, $5 \times 10^3$, or $10^4$ CD34$^+$ cells, respectively, showed donor-derived lymphocytes and granulocytes at a level that was directly proportional to the number of CD34$^+$ cells injected (Table 3). In mice injected with $10^5$ CD34$^+$ cells, 59% ± 15% of PB leukocytes were of donor origin and were comprised of 69% B cells (B220$^+$), 31% T cells (Thy-1$^+$), and 3% myeloid (Gr-1$^+$, Mac-1$^+$) cells. The frequency of CRU derived from this data was 1 per 2,465 (95% confidence limits, 1 per 1,612 to 1 per 5,236) CD34$^+$ BM cells. We could not determine the frequency of CRU among CD34$^+$ BM cells without further fractionation because their degree of contamination (≈2%) by CD34$^+$ cells would be sufficient to account for any stem cell activity observed at doses of ≥$10^5$ cells (which would contain 2,000 CD34$^+$ cells). At present, our results suggest only that CRU represent less than 1 per $5 \times 10^4$ CD34$^-$ BM cells. Additional studies using more enriched HSCs are in progress to address this issue in more detail.

Table 3. Multilineage Competitive Repopulation of Lethally Irradiated Mice With CD34$^+$ BM Cells

<table>
<thead>
<tr>
<th>Cells Injected</th>
<th>No. of Mice Positive</th>
<th>% Donor (mean ± SD)</th>
<th>B220</th>
<th>Thy-1</th>
<th>Gr-1/Mac-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^4$ CD34$^-$</td>
<td>0/10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>$5 \times 10^4$ CD34$^-$</td>
<td>0/8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>$2 \times 10^5$ CD34$^+$</td>
<td>3/8</td>
<td>5 ± 7</td>
<td>58 ± 36</td>
<td>21 ± 35</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>$5 \times 10^5$ CD34$^+$</td>
<td>8/10</td>
<td>21 ± 18</td>
<td>53 ± 12</td>
<td>31 ± 14</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>$10^6$ CD34$^+$</td>
<td>15/15</td>
<td>59 ± 15</td>
<td>69 ± 10</td>
<td>31 ± 9</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

CD34$^-$ and CD34$^+$ cells were sorted from BAL (Ly-5.2$^+$) BM and injected together with $2 \times 10^6$ "compromised" Ly-5.1 BM cells into lethally irradiated Ly-5.1 hosts. PB was analyzed 4 months later. Shown are the number of positive mice with ≥5% Ly-5.2$^+$ leukocytes and the percentage of donor cells expressing B220, Thy-1, or Gr-1/Mac-1 antigens.

Abbreviation: NA, not applicable.
DISCUSSION

The aim of this study was to determine whether a recently developed antimurine CD34 MoAb, RAM34, could be used to isolate the entire primitive stem and progenitor cell compartment of murine BM. In contrast to human BM cells (average, 1.5% CD34), CD34 was expressed on 4% to 17% of murine BM cells at intermediate (≈80%) or high (≈20%) levels (Fig 1). The majority of CD34+ BM cells showed a lymphoblastoid morphology, and approximately one third expressed lineage-associated antigens such as CD4, CD8, Gr-1, Mac-1, B220 and Thy-1. Of these, CD4, Mac-1, Gr-1 and Thy-1 are expressed at low levels on some pluripotent hematopoietic progenitors, suggesting that CD34 will be a useful marker for the further enrichment of murine hematopoietic stem cells.

Sca-1+ Thy-1+ Lin- BM cells were also heterogeneous with respect to CD34 expression, with ≈15% being CD34+ (Fig 2). Sca-1+ Thy-1+ Lin- cells are 1,000-fold enriched in hematopoietic cells able to confer radioprotection and form d.12 spleen colonies with near unit efficiency. In addition, fewer than 50 Sca-1+ Thy-1+ Lin- BM cells can repopulate both lymphoid and myeloid lineages when transplanted into lethally irradiated mice. Several studies have shown that this HSC population can be subdivided with respect to macrophage and c-kit expression or retention of the supravital fluorescent dye, Rhodamine-123. The mac-1+CD4+ c-kit+ or Rh-123+ subpopulations of Sca-1+ Thy-1+ Lin- BM are enriched for stem cells with long-term reconstitution potential, whereas the mac-1+CD4-, mac-1+CD4+ or Rh-123+ subpopulations provide only transient reconstitution in vivo. In light of these observations, we have initiated experiments to test the repopulating potential of the CD34- and CD34+ fractions of Sca-1+ Thy-1+ Lin- BM to determine if they are also functionally different (F.M., manuscript in preparation). Interestingly, most Sca-1+ Thy-1+ Lin- cells expressed lower levels of CD4 than some Sca-1- or Thy-1- cells (Fig 2). This suggests that selection of CD34-bright cells could result in a loss of some HSC activity, and is in opposition with the description of high levels of CD34 on the most primitive human HSCs. The reasons for this are unclear but may reflect differences in the staining observed with different anti-CD34 reagents or species- or ontogeny-related variations in CD34 expression. In this regard, the RAM34 MoAb should facilitate a more direct analysis of the developmental potential of murine cells expressing different levels of CD34 than that possible with polyclonal antisera. 

Consistent with previous studies of the expression of CD34 on human and murine hematopoietic cells, we found that the majority of CFCs and CFU-S in B.A1 mice expressed CD34. Consequently, isolation of CD34+ BM cells enabled a sixfold to 10-fold enrichment of BFU-E, CFU-GM, and CFU-GEMM and a 4.5-fold enrichment of d.8 and d.12 CFU-S (Table 2). Only 3% of CFU-GM and 5% of d.8 CFU-S were recovered in the CD34- population. Slightly more d.12 CFU-S (12%) were recovered in this fraction, which is consistent with the absence of CD34 on ≈15% of Sca-1+ Thy-1+ Lin- BM cells that are highly enriched in d.12 CFU-S. Early B-cell precursors able to proliferate on Sy5-1 stromal cells were recovered in both the CD34+ and CD34- populations. However, the large number of primitive pro-B and pre-B cells generated in 3 week cultures of CD34- BM showed a more profound enrichment of B progenitors in this fraction (Fig 3). A smaller but significant number of pro-B and pre-B cells were transiently generated by CD34- cells. These results suggest that CD34 antigen is lost at an early stage in B lymphopoiesis, and are in accordance with the human B-cell differentiation pathway in which CD34 decreases during the transition from pro-B to pre-B cells. Nevertheless, the inability of up to 5 x 10^6 CD34- cells to repopulate B lymphopoiesis in vivo suggests either a much lower frequency of B progenitors or a lower proliferative and/or competitive potential of these cells as compared with those in the CD34+ population. Consistent with human studies, only CD34- BM cells could generate mature CD3+TCRαβ T cells in thymic reconstitution assays in SCID mice (Fig 4). As with B-cell production, the threefold to sevenfold greater and more prolonged production of T cells generated by CD34+ versus unfractionated BM is consistent with the enrichment of more immature T-lymphoid progenitors among CD34- cells.

Only CD34- BM cells could rescue mice from the lethal effects of total-body irradiation at the cell dose tested. Most mice (79%) injected with 10^5 CD34- cells survived at least 45 days, whereas all recipients of 10^6 CD34- cells died by day 21. To determine if CD34 was expressed on more primitive stem cells that become active very late after transplantation and, therefore, may not be radioprotective, we used a competitive long-term repopulation assay. Limiting dilution experiments established that 1 per 2,465 CD34+ BM cells was able to repopulate B-and T-lymphoid, and myeloid lineages for at least 4 months. After this time, mice competitively repopulated with 10^4 CD34- cells showed a mean of 59% donor-derived PB leucocytes (Table 3). A similar level of engraftment was previously reported with 300 Sca-1+ Thy-1+ Lin- BM cells. Our observation that ≈1.5% of CD34- BM cells are Sca-1+ Thy-1+ Lin- suggests that 10^4 CD34- cells should contain ≈150 Sca-1+ Thy-1+ Lin- cells and therefore fits well with published findings. None of the mice competitively transplanted with up to 5 x 10^4 CD34- BM cells showed ≈5% donor-type cells. However, we cannot exclude the presence of a rare population of CD34- stem cells, as suggested by the lack of CD34 on ≈15% of Sca-1+ Thy-1+ Lin- BM (Fig 2). Such HSCs may have been sufficiently diluted by mature cells to prevent their detection after injection of 5 x 10^4 CD34- BM cells. Indeed, our calculations indicate that Sca-1+ Thy-1+ Lin- BM cells represent only ≈0.02% of CD34- BM, so that 5 x 10^4 CD34- cells would contain at most 10 of these phenotypically defined HSCs. This dose is significantly below the ≈45 cells determined by limiting dilution experiments to contain at least one functional long-term CRU. However, because the ≈2% contamination of CD34- BM by CD34+ cells is sufficient...
to account for any functional activity among greater than $5 \times 10^4$ CD34$^+$ cells (ie, $10^5$ CD34$^+$ cells contain 2,000 CD34$^+$ cells that repopulated 38% of mice), a more detailed examination of the long-term repopulating potential of CD34$^-$ cells will require an analysis of populations that are already highly enriched in HSCs. Significantly, our current studies indicate that CD34$^-$ Sca-1$^+$ Thy-1$^+$ Lin$^-$ cells may be more potent on a per cell basis than their CD34$^+$ counterparts and contain a higher frequency of CRU (F.M., manuscript in preparation).

Overall, it appears that CD34 is a primitive hematopoietic cell antigen in a similar sense as Sca-1, Thy-1 or c-kit. As with Sca-1 and Thy-1, the function of CD34 is unknown. Proteolytic cleavage of CD34 does not affect the ability of human hematopoietic progenitors to proliferate in vitro. Rather, a role for CD34 in the compartmentalization of HSCs is suggested by the increased adherence of murine hematopoietic cells to human, but not murine, BM stromal cells when murine progenitors have been engineered to ectopically express human CD34. Analysis of hematopoiesis in CD34 knock-out mice indicates that CFU-S and CFC numbers in the yolk-sac, fetal liver, and adult BM and spleen are reduced twofold to threefold. CD34-deficient progenitors are also unable to proliferate in liquid cultures in response to hematopoietic growth factors. Interestingly, however, BM and PB differential counts and vascular development appears normal, and the recovery of circulating leukocytes, erythrocytes, and platelets after sublethal irradiation was not different in CD34-null and wild-type animals. This finding indicates that CD34$^-$ mice retain an expandable pool of hematopoietic progenitor cells and suggests a redundancy in CD34 function.

In conclusion, we find that the RAM34 antimurine CD34 MoAb is a useful tool for the fractionation and enrichment of both primitive HSCs and more mature progenitor cells. In this regard, CD34 represents an additional powerful marker for the isolation of murine hematopoietic cells and should facilitate future comparisons of the biology of CD34-selected human and murine stem cells.

NOTE ADDED IN PROOF

We acknowledge that, while this report was in press, Osawa et al reported the presence of primitive stem cells with long-term repopulating potential in a CD34$^{-}$Lin$^-$ fraction of murine bone marrow that were pre-enriched for c-kit$^+$Sca-1$^+$Lin$^-$ cells.

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