Expression of Thy-1 on Human Hematopoietic Progenitor Cells

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Summary

Expression of Thy-1 on hematopoietic cells from human fetal liver (FL), cord blood (CB), and bone marrow (BM) was studied with a novel anti-Thy-1 antibody, 5E10. Specificity of 5E10 for human Thy-1 was demonstrated by immunoprecipitation of a 25–35-kD molecule, and the sequence of a cDNA that was cloned by immunoselection of COS cells transfected with a cDNA library derived from a $5E10^+$ cell line. Two- and three-color immunofluorescence staining experiments revealed that the Thy-1 expression is restricted to, on average, 1–4% of FL, CB, and BM cells, and binding to these cell types is essentially restricted to a very small subset of lymphoid cells and ~25% of $CD34^+$ cells. $Thy\text{-}1^+CD34^+$ cells were further characterized as $CD38^{lo}/CD45RO^+/CD45RA^-/CD71^{lo}/c\text{-}kit^{lo}$ and rhodamine 123^{dull}. When $CD34^+$ cells were sorted on the basis of Thy-1 expression, the majority of clonogenic cells were recovered in the $CD34^+Thy\text{-}1^-$ fraction, whereas the majority of cells capable of producing myeloid colonies after 5–8 wk of long-term culture (long-term culture initiating cells) were recovered in the $Thy\text{-}1^+CD34^+$ fraction. In addition to $CD34^+$ cells, Thy-1 was found to be expressed on a variable, very small number (<1%) of $CD34^-$ mononuclear cells in BM, CB, and peripheral blood that were further characterized as $CD3^+CD4^+$ lymphocytes. The restricted expression of Thy-1 on primitive hematopoietic cells is in agreement with a previous report (Baum et al., 1992. *Proc. Natl. Acad. Sci. USA.* 89:2804) in which Thy-1 expression was used to enrich for primitive hematopoietic cells from fetal tissue. Compared with those previous studies, we found Thy-1 expression on a larger proportion of $CD34^+$ cells (25% in our study vs. 5% in Baum et al.) and furthermore performed studies on Thy-1 expression on $CD34^+$ cells from CB, FL, and BM in relation to markers that are known to be differentially expressed on hematopoietic cells. Taken together our results indicate that Thy-1-specific antibody 5E10 is an attractive tool for further studies on the biology and purification of human stem cells.

The hematopoietic system consists of a highly heterogeneous mixture of cells at various stages of differentiation. The majority of these cells have limited life spans and limited or no proliferative potential. Cell number is maintained through the proliferation of a small number of pluripotent stem cells and their immediate progeny, the cells of the progenitor cell compartment (1). Both stem cells and their progeny express CD34, a highly glycosylated cell surface protein present on 1–4% of low density bone marrow (BM)[1] mononuclear cells (2). mAbs against CD34 have been the primary tool used for the purification of human stem cells, either in immunoadsorption (panning) procedures, immunoabsorbance columns, magnetic separations, or in flow cytometry (reviewed in reference 3). $CD34^+$ cells are highly heterogeneous both functionally and in the markers they express; therefore, additional mAbs have been used to subdivide the CD34 compartment. Most current techniques for further purification of the most primitive $CD34^+$ cells have relied on negative selection. Antibodies against various lineage markers (4), transferrin receptor (CD71) (5, 6), the high molecular weight isoform of the human leukocyte antigen (CD45RA) (6, 7), CD33 (8, 9), CD38 (10), and HLA-DR (5, 11), have been used by different groups for this purpose. We have investigated the use of novel mAbs raised against the human erythroid leukemia hematopoietic cell line HEL (12) for the further subdivision of cells that express CD34.

[1] *Abbreviations used in this paper:* BM, bone marrow; CB, cord blood; CFC, colony-forming cells; FL, fetal liver; HFN, Hank's Hepes buffered salt solution containing 2% FCS and 0.1% sodium azide; HPP, high proliferative potential; LTC-IC, long-term culture initiating cells; NHS, normal human serum; NSS, normal sheep serum; PB, peripheral blood; PI, propidium iodide; PO, peroxidase; SA-HRPO, Streptavidin-conjugated horseradish peroxidase; SA-RPE, Streptavidin-R phycoerythrin; TNP, trinitophenol.

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One mAb, 5E10, was found to react with a minor population of BM cells (1–4%), including ~25% of $CD34^+$ cells and a small subset of lymphoid cells. Further experiments showed that the subpopulation of $CD34^+$ cells recognized by 5E10 is highly enriched for phenotypically and functionally very primitive hematopoietic precursor cells and that the molecule recognized by 5E10 is Thy-1. In view of this finding and a recent report indicating that Thy-1 is expressed on a candidate human hematopoietic stem cell population from fetal BM (4), we wished to correlate expression of Thy-1 on $CD34^+$ cells from adult BM in relation to markers that are known to be preferentially expressed on functionally distinct hematopoietic subpopulations of such cells as well as $CD34^+$ cells derived from fetal liver (FL) and umbilical cord blood (CB).

Materials and Methods

Cells. Heparinized BM samples were obtained from informed and consenting individuals donating for allogeneic transplantation. CB samples were obtained from the umbilical cord at the time of birth from full-term pregnancies (kindly provided by Dr. J. O'Toole and his colleagues at the Royal Columbian Hospital, Coquitlam, BC). Peripheral blood (PB) samples were donated by healthy consenting volunteers. FL cells were obtained from elective, therapeutic abortions, in the 12–20th week of gestation. Low density mononuclear cells (<1.077 g/cm^3) were isolated by density separation using Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden). Interphase cells were removed and washed twice in Hank's Hepes buffered salt solution containing 2% FCS and 0.1% sodium azide (HFN) before being resuspended at 10^7 cells/ml in HFN + 5% normal human serum (NHS) to block F_c receptors (F_cR). For some experiments, RBC were lysed by mixing samples with a 10-fold excess of ammonium chloride lysing solution. Samples were incubated for 15 min on ice, pelleted, and washed twice in HFN.

HEL, K562, KG1a, DHL4, CCRF-CEM, MOLT4, HUT78, and Sp2/OAg14 (a murine myeloma line) cell line cells were all obtained from American Type Culture Collection (Rockville, MD) and grown in DME supplemented with 10% FCS and 5×10^{-5} M 2-ME (DME 10).

Antibodies. IgG1 mAbs specific for CD34 (8G12), CD71 (OKT9), CD45RA (8D2), CD38 (THB7), and 5E10 were purified from tissue culture supernatants using protein G (Pharmacia LKB). 8G12 labeled with cyanine 5 was previously described (6), and used at 10 μg/ml. OKT9, 8D2, and THB7 were labeled with FITC (F-7250; Sigma Chemical Co., St. Louis, MO), and were used at 1, 2, and 2 μg/ml, respectively. 5E10 was labeled either with fluorescein (5 and 6-carboxyfluorescein succinimydyl ester; Molecular Probes, Eugene, OR) or biotin (biotin xx succinimydyl ester; Molecular Probes) and used at 8 and 4 μg/ml, respectively. Ascitic fluid containing 17F11, an anti-c-*kit* Ab (IgM), was obtained from AMAC Monoclonal Antibodies (Westbrook, ME) and was used at a dilution of 1:1,000. Biotinylated goat anti–mouse IgG (biotin-anti-mIg, 115–065–062; Jackson, Westgrove, PA) was diluted 200× in HFN containing 5% normal sheep serum (NSS) and 5% NHS.

Generation of 5E10. Female BALB/c mice were injected with 10^7 HEL cells suspended in CFA. Mice were boosted with 10^7 HEL cells 4 wk later, 4 d before the fusion was performed. Spleens were disaggregated to form a single-cell suspension, and cells fused at a ratio of five spleen cells to one Sp2/0 Ag14 cell. Hybridomas were plated in DME 10 + hypoxanthine, aminopterin, and thymidine (HAT) + 50 U/ml IL-6 (R & D Systems, Minneapolis, MN). After 7 d, media were removed and fresh DME 10 + hypoxanthine, thymidine + 10 U/ml IL-6 was added. Hybridoma supernatants were screened on day 13 or 14 by ELISA as previously described (13). Briefly, 60-well Terasaki plates were coated with low density PBL or HEL cells. Plates were incubated with undiluted hybridoma supernatants, followed by a peroxidase-conjugated sheep anti–mouse secondary antibody (Dimension Laboratories, Mississauga, Ontario), and finally with the substrate solution containing *O*-phenylenediamine dihydrochloride (OPD; Sigma Chemical Co.). Hybridomas producing Abs that reacted with HEL cells, but not PBL, were then tested by flow cytometry on low density BM mononuclear cells for reactivity with $CD34^+$ cells. Hybridomas were cloned by plating cells in semisolid methylcellulose and plucking colonies. Clones were selected by ELISA on HEL cells.

Flow Cytometry. All staining procedures were performed at a cell concentration of 10^7/ml. Indirect staining techniques were used for most experiments. Cells were incubated with 5E10 or control IgG1 antibody specific for either trinitrophenol (TNP) or peroxidase (PO) at 4°C for 30 min, followed by two washes with HFN. Samples were resuspended in biotin-anti-mIg (1:200 dilution in HFN + 5% NSS + 5% NHS), incubated for 30 min at 4°C, washed twice in HFN, and resuspended in a half volume of an irrelevant mAb (100 μg/ml of mIgG1 anti-TNP in HFN) to block any residual biotin anti-mIg. Samples were incubated for 10 min at 4°C. Directly conjugated mAbs and Streptavidin-R phycoerythrin (SA-RPE; Molecular Probes) were then added and cells incubated for an additional 30 min at 4°C, washed twice in HFN, and resuspended in HFN + propidium iodide (PI; p-5264; Sigma Chemical Co.) at 1 μg/ml. Alternatively, cells were labeled with either directly labeled (5E10-FITC) or biotinylated 5E10 followed by SA-RPE or avidin-FITC (3 μg/ml; Becton Dickinson & Co., San Jose, CA). c-*kit*-stained samples were incubated with 17F11 (1:1,000 ascites) in the first step, followed by biotin anti-mIg, and SA-RPE. Staining was then amplified by two successive rounds of incubation with a biotinylated anti-RPE mAb (1 μg/ml) followed by 2 μg/ml SA-RPE (14). Parallel samples were stained with an irrelevant IgM antibody.

Rhodamine 123 (Rh123)-stained samples were labeled with Rh123 before Ab staining. Cells were resuspended in HF + Rh123 (Molecular Probes) at 0.1 μg/ml, incubated for 20 min at 37°C, and washed twice in HF. Samples were then split and incubated for 20 min at either 4 or 37°C, washed twice in HFN, and resuspended in HFN + 5% NHS for Ab staining. Analysis and/or sorting of cells was performed on a FACStar Plus® (Becton Dickinson & Co.) equipped with a 5-W Argon and a 30-mW helium neon laser (Spectra-Physics, Mountain View, CA).

Staining of $CD34^-$ $5E10^{2+}$ Cells with Lineage Markers. Anti-Leu-4 (anti-CD3), anti-Leu-2a (anti-CD8), anti-Leu-3a (anti-CD4), anti-Leu-19-PE (anti-CD56), and anti-Leu-12-FITC (anti-CD9) were obtained from Becton Dickinson & Co.). M110 (anti-CD13), M138 (anti-CD14), and M112 (anti-CD9) ascitic fluids were part of the myeloid panel from the Fourth International Leukocyte Typing Workshop and were used at a 1:1,000 dilution. Cells were incubated with unlabeled primary Ab at 4°C for 30 min, washed twice in HFN, and resuspended in a 1:100 dilution of FITC-conjugated sheep anti–mouse (SAM-FITC; Jackson Immunoresearch Laboratories, West Grove, PA) in HFN + 5% NHS + 5% NSS. Samples were incubated for 30 min at 4°C, washed twice in HFN, resuspended in irrelevant mouse IgG, and incubated for 10 min at 4°C to block residual SAM-FITC. Samples were then labeled with biotinylated Abs (5E10-b or mIgG-b), followed by SA-RPE or A-FITC, 8G12-Cy5, PI, and directly conjugated mAbs.

Long-Term Cultures and Progenitor Assays. Long-term cultures and progenitor assays were performed as previously described (11), except for the addition to methylcellulose cultures of 20 ng/ml of the GM-CSF/IL-3 fusion protein (15) and 50 ng/ml human mast cell growth factor (MGF; *c-kit* ligand) (16) which were both kindly provided by Dr. D. E. Williams (Immunex, Seattle, WA). Sorted cells were plated in long-term cultures and methylcellulose progenitor assays. Methylcellulose colonies were counted on day 14 to determine the number of colony-forming cells (CFC) in the original sample, and at day 28 to determine the number of high proliferative potential CFC (HPP-CFC) (17). HPP-CFC are late-appearing, large (>1 mm in size) densely packed colonies composed of >50,000 cells. Colonies are primarily composed of granulocytes although some monocytes may be present. Long-term cultures were harvested at 5 and 8 wk, and colony assays performed to determine the number of long-term culture-initiating cells (LTC-IC) present in the original samples (11). The number of LTC-IC present initially is linearly related to the number of clonogenic cells present after 5–8 wk, with LTC-IC producing an average of four clonogenic cells in standard methylcellulose cultures (18).

5E10 Immunoprecipitation. DHL4 ($5E10^+$) and CCRF-CEM ($5E10^-$) cells were washed twice in HBSS, and 10^7 cells were lysed at a final concentration of 2×10^7 cells/ml in 0.5% NP-40, 50 mM Hepes, 100 mM NaF, 100 mM NaPi, 2 mM Na_3VO_4, 2 mM EDTA + 2 μg/ml leupeptin, 5 mM PMSF (Sigma Chemical Co.), and 100 Kallikrein inhibitor U/ml aprotinin, pH 7.5. Lysed samples were then microfuged for 10 min at 10,000 rpm to remove cell debris, and supernatants were transferred to new tubes. Cell lysates were precleared by two successive rounds of incubation with anti-TNP-bound protein A–Sepharose beads (Sigma Chemical Co.). Anti-TNP (an irrelevant IgG1 control mAb) was added at 5 μg/ml to protein A–Sepharose beads and incubated for 30 min at 4°C. 150 μl of this Ab/bead mixture was added to cell lysates, samples were mixed at 4°C on a Nutator for 60 min, and beads were pelleted by microfuging at maximum speed for 1 min. Supernatants were transferred to new tubes, and the preclearing was repeated. For immunoprecipitation, unlabeled 5E10 or an isotype-matched control Ab (anti-TNP) was added at a final concentration of 5 μg/ml to the cleared lysates, and samples were mixed on a Nutator for 30 min at 4°C. 20 μl of protein A–Sepharose beads was then added to each tube, and samples were mixed on a Nutator for 60 min at 4°C. The beads were then pelleted and washed three times in low salt buffer (0.2% NP-40, 2 mM EDTA, 10 mM Tris, pH 7.5, 150 mM NaCl), twice in high salt buffer (0.2% NP-40, 2 mM EDTA, 10 mM Tris, pH 7.5, 500 mM NaCl), and once in 10 mM Tris. 4× SDS sample buffer (30% glycerol, 25% 2-ME, 9.2% SDS, 0.8% bromphenol blue), and dH_2O were added to a final volume of 160 μl and samples were boiled for 2 min. Proteins were separated on a 20-cm 15% SDS-PAGE gel and were electroblotted to Immobilon P (Millipore, Mississauga, Ontario). The Immobilon membrane was blocked by incubating for 2 h at room temperature in blocking solution. Bands were then electroblotted to an Immobilon membrane using a transblot apparatus (Bio-Rad Laboratories, Richmond, CA). The membrane was incubated for 2 h at room temperature in blocking solution (2% BSA, 0.05% NaN_3, 0.05% Tween in PBS), followed by three 5-min rinses in wash solution (0.1% BSA, 0.05% Tween in PBS). The membrane was then transferred to a solution containing 0.5 μg/ml 5E10 biotin in wash solution, incubated for 30 min at room temperature, and rinsed three times in wash solution. The membrane was then incubated for 60 min in a solution of Streptavidin-conjugated horseradish peroxidase (SA-HRPO; Bio-Can, Mississauga, Ontario) (1:20,000 in wash solution), rinsed four times in wash solution, and once in PBS. The blot was developed using enhanced chemiluminescence (ECL) substrate solution (Amersham, Oakville, Ontario) according to manufacturer's instructions and exposed for 2 min to Kodak Xomat AR film.

cDNA Immunoselection and Sequencing. cDNA was synthesized from HEL cell mRNA as described (19) and cloned into pAX142, which is a variant of the pAX114 expression vector (20). Plasmid DNA of the cDNA library was transfected into COS-1 cells (21) by electroporation (20). 3 d later, the transfected cells were treated with 5E10 mAb and then panned on dishes coated with goat anti–mouse IgG. Plasmids were then recovered from the adherent cells by alkaline lysis, recloned by transformation of *Escherichia coli*, and retransfected into COS cells. These procedures have been described in detail previously (20).

The sequence of the cDNA was determined by the chain termination method, using Sequenase enzyme and procedures supplied by United States Biochem. Corp. (Cleveland, OH). Sequence analysis and data base searching were performed with the GCG software package (22).

Results

Generation of mAb 5E10. Spleen cells from mice immunized with HEL cells were fused with Sp2/0 myeloma cells and plated in selective media. Approximately 1,000 hybridoma supernatants were screened for reactivity with HEL cells and PBL by ELISA. 12 supernatants appeared HEL specific by ELISA and reactivity was confirmed by flow cytometry. Low density BM mononuclear cells were double stained with supernatants of cloned hybridomas and 8G12-FITC (anti-CD34) to examine reactivity with $CD34^+$ cells. Gates were set as shown in Fig. 1 to exclude dead (PI^+) and high side scatter (SSC) cells. mAb 5E10 labeled a minority of BM cells, including a subpopulation of $CD34^+$ cells, and was selected for further study. It was difficult to discriminate between 5E10- and control-stained samples without gating on $CD34^+$ cells (Fig. 2, *A* and *B*). Approximately 25% of the cells that were CD34 bright were $5E10^+$ (Fig. 2, *C* and *D*). The determinant recognized by 5E10 is apparently expressed at very low levels as normal cells stained with directly conjugated 5E10 reagents were weakly stained (Fig. 2, *E* and *G*) even though certain cell lines were very brightly labeled by identical reagents.

5E10 Immunoprecipitates a 25–35-kD Protein. Several human hematopoietic cell lines were examined for 5E10 staining. CCRF-CEM, MOLT4, and HUT78 are T cell lines, DHL4 is a B cell lymphoma line, and K562, HEL, and KG1a are all leukemic lines with various characteristics of early hematopoietic blast cells. HEL, DHL4, and HUT78 were brightly stained by 5E10, MOLT4 only weakly, and K562 and CCRF-CEM not at all. Only a few percent of KG1a cells were $5E10^+$, but staining was weak (data not shown). From these results, it was decided to use DHL4 cells (brightest staining) and CCRF-CEM (lowest background staining) for immunoprecipitation with 5E10. DHL4 (Fig. 3, lanes *1* and *2*) and CCRF-CEM (Fig. 3, lanes *3* and *4*) cells were lysed and immunoprecipitated with either 5E10 (Fig. 3, lanes *1* and *3*) or an isotype-matched irrelevant control antibody (Fig. 3, lanes *2* and *4*). Immune complexes were then run on 15%

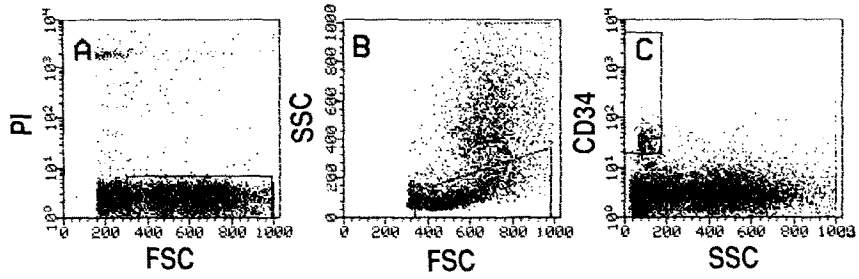

Figure 1. Gates used for analysis of antibody reactivity with BM cells. BM cells were gated to exclude dead cells (*A*), and include cells with low side scatter (*SSC*) and low to medium forward light scatter (*B*, *FSC*), and low side scatter and high CD34 expression (*C*). The same gates were used for CB and FL samples.

SDS-PAGE and blotted to an Immobilon membrane. Membranes were incubated with biotinylated 5E10 and SA-HRPO, developed by incubating in chemiluminescence solution, and exposed to film. Several weak bands were seen in all lanes, but a single broad band at 25–35 kD was specific for the 5E10-immunoprecipitated DHL4 lysates (Fig. 3, lane *1*).

Expression Cloning of a cDNA Encoding the 5E10 Antigen. A cDNA encoding the 5E10 antigen was isolated by immunoselection of a cDNA library expressed in transfected COS cells. cDNA was synthesized from HEL cell mRNA and ligated into the pAX142 expression vector. Pooled plasmid DNA of the cDNA library was transfected into COS-1 cells, which were selected for the acquisition of antigen expression by panning with the 5E10 mAb. Plasmids were then isolated from the adherent cells, amplified by transformation into *E. coli*, and retransfected into COS cells. After two such rounds of transfection and panning, individual plasmid clones recovered from the selected cells were tested by FACS® for their ability to transfer 5E10 reactivity to COS cells. A single clone with a 2,000-bp cDNA insert (pSL307c18) produced strong and specific reactivity with the 5E10 mAb. Sequence was obtained from both ends of the pSL307c18 cDNA and is shown for the 5′ end in Fig. 4. A search of the GenBank database revealed a complete identity with the Thy-1 cDNA (23). Restriction maps of the pSL307c18 and Thy-1 cDNAs were matched throughout their overlapping regions, indicating that they were identical except for the extended length of the 5′ and 3′ noncoding regions of the pSL307c18 cDNA.

Functional Analysis of CD34⁺ Cells from BM Differing in Thy-1 Expression. Low density BM cells were labeled with 5E10 and 8G12 (anti-CD34), sorted into Thy-1⁻CD34⁺ and Thy-1⁺CD34⁺ populations, and plated in long-term

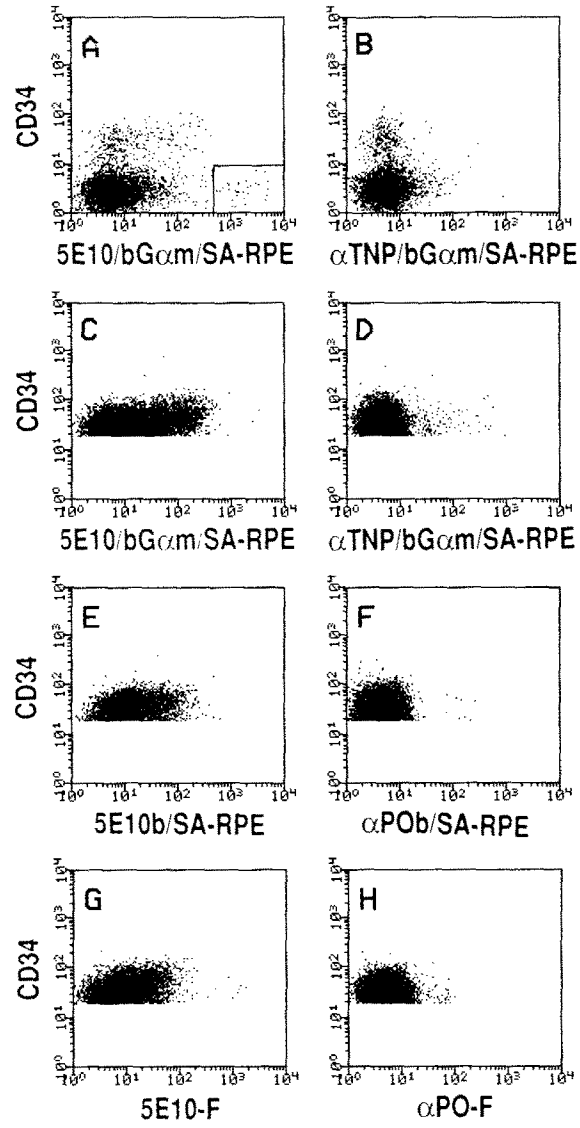

Figure 2. Direct vs. indirect staining of cells with 5E10. Low density BM mononuclear cells were labeled with 5E10 or isotype-matched control antibody (anti-TNP or anti-PO) together with antibodies against CD34. 5E10 stains a small population of CD34⁻ cells (*A*, *box*) and 20–30% of CD34⁺ cells. Indirect staining results in the brightest staining but also the highest background. Note that 5E10 appears to react preferentially with CD34 bright cells.

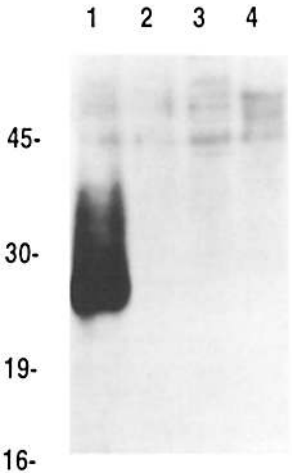

Figure 3. 5E10 immunoprecipitates a broad band ~25 kD. DHL4 (5E10⁺) and CCRF-CEM (5E10⁻) cells were immunoprecipitated with either 5E10 or anti-TNP (an irrelevant isotype-matched control antibody) and Western blotted with 5E10. Lane *1*, DHL4 cells immunoprecipitated with 5E10; lane *2*, DHL4 cells immunoprecipitated with anti-TNP; lane *3*, CCRF-CEM cells immunoprecipitated with 5E10; lane *4*, CCRF-CEM cells immunoprecipitated with anti-TNP. The very strong band ~25 kD was specific for DHL4 cells, and was not precipitated by the control antibody.

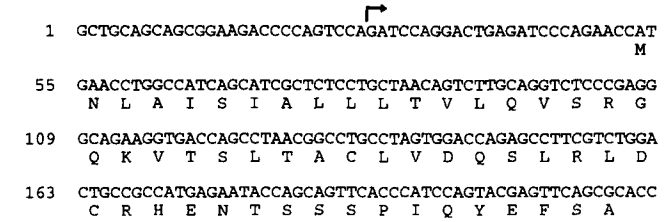

Figure 4. Sequence of the 5' end of the cDNA encoding 5E10 antigen. The first 216 bases of the pSL307c18 cDNA are shown, reading from the 5' end of the cDNA. The arrow marks the beginning of identity with the published human Thy-1 sequence (25) (GenBank accession no. M11749), which starts at this position. The translation beginning at the first initiation codon is shown below the sequence.

cultures and progenitor assays. Sort windows were set to give a clear separation of Thy-1$^+$ and Thy-1$^-$ cells; therefore, some 5E10 weakly stained cells were excluded, resulting in poor recoveries for some experiments (Tables 1 and 2). In three of four experiments, myeloid and erythroid CFC were depleted in the Thy-1$^+$ fraction while the majority of these CFCs were recovered in the Thy-1$^-$ fraction (Table 1). HPP-CFC, a more primitive progenitor cell that can be assayed in semisolid medium (5), was highly enriched in the Thy-1$^+$ fraction (30–160-fold; Table 1) and were either absent from or only moderately enriched in the Thy-1$^-$ fraction (Table 1). Similarly, week 5 and 8 LTC-IC (18) were highly enriched (48–644- and 72–644-fold, respectively) in the Thy-1$^+$ fraction (Table 2). These results indicate that Thy-1 expression is highest on week 8 LTC-IC, followed by week 5 LTC-IC and HPP-CFC, and is absent from the majority of mature CFC.

Phenotypic Analysis of Thy-1$^+$CD34$^+$ Subpopulations in BM, CB, and FL. Thy-1$^+$CD34$^+$ cells were further characterized by multiparameter flow cytometry. CB and FL samples were included to examine whether Thy-1 expression is altered during ontogeny. Low density mononuclear cells from BM, CB, and FL were labeled in the same experiments with 5E10, 8G12 (anti-CD34), and either Rh 123 or mAbs specific for c-*kit*, CD38, CD71, or CD45RA. Dead cells were excluded by gating out PI$^+$ cells. Additional gates were set to exclude cells with high SSC and to restrict analysis to cells with medium to high forward scatter (FSC), and high CD34 expression (Fig. 1). Profiles for BM, CB, and FL CD34$^+$ cells are shown in Figs. 5 and 6 and are summarized in Table 3. Again, without further gating it was difficult to discriminate 5E10 staining from background staining (compare Fig. 5, *A*, *E*, and *I* with *B*, *F*, and *J*). Up to 50% of CD34$^+$ cells from FL were Thy-1$^+$ (Fig. 5 *D*), and this population was smaller but clearly present in CD34$^+$ cells from CB and BM (Fig. 5, *H* and *L*). Note that CD45RA expression is low on FL cells, high on some CB and BM cells, and that Thy-1 expression appears restricted to CD34$^+$ CD45RA$^-$ cells in adult BM but includes some CD34$^+$CD45RA$^+$ cells from CB (Fig. 5, *D*, *H*, and *L*).

c-*kit* expression was restricted to a few percent of BM, CB,

Table 1. *Recovery and Enrichment of Colony-forming Cells over CD34$^+$ Cells Separated on the Basis of Thy-1 Expression*

			BFU-E			CFU-GM			HPP-CFC		
Exp.	Fraction sorted	Percent sorted	Frequency	Enrichment	Percent recovery	Frequency	Enrichment	Percent recovery	Frequency	Enrichment	Percent recovery
			per 10^5			*per 10^5*			*per 2 × 10^6*		
1	Unsorted	100	575	1	100	455	1	100	366	1	100
	Thy-1$^-$CD34$^+$	2.1	10,750	19	39	13,750	30	63	2,500	6.8	14
	Thy-1$^+$CD34$^+$	0.56	5,750	10	5.6	17,625	38	21	27,500	75	42
2	Unsorted	100	830	1	100	580	1	100	2,000	1	100
	Thy-1$^-$CD34$^+$	2.6	19,900	24	62	5,500	9.5	25	0	0	0
	Thy-1$^+$CD34$^+$	0.39	500	0.6	0.2	7,400	13	5	60,000	30	12
3	Unsorted	100	258	1	100	205	1	100	360	1	100
	Thy-1$^-$CD34$^+$	1.5	9,750	38	53	8,750	43	64	0	0	0
	Thy-1$^+$CD34$^+$	0.48	9,500	37	18	24,000	117	56	57,500	160	76
4	Unsorted	100	153	1	100	186	1	100	720	1	100
	Thy-1$^-$CD34$^+$	1.4	4,600	30	42	8,500	46	64	10,000	13.8	19
	Thy-1$^+$CD34$^+$	0.15	4,500	29	4.4	7,750	42	6.2	60,000	83	12.5

BM cells were sorted on the basis of Thy-1 labeling and CD34 expression, and placed in methylcellulose cultures. Colony-forming cells (CFU, BFU) were scored on day 14, and HPP-CFC on day 28. Enrichments were calculated by dividing the frequency of CFU in sorted fractions by the frequency for unsorted PI$^-$ cells. Recoveries were calculated by multiplying the enrichment by the percent sorted.

Table 2. *Recovery and Enrichment of LTC-IC over CD34⁺ Cells Separated on the Basis of Thy-1 Expression*

Exp.	Fraction sorted	Percent sorted	Week 5 LTC-IC Frequency	Week 5 LTC-IC Enrichment	Week 5 LTC-IC Percent recovery	Week 8 LTC-IC Frequency	Week 8 LTC-IC Enrichment	Week 8 LTC-IC Percent recovery
			per 2 × 10⁶			*per 2 × 10⁶*		
1	Unsorted	100	775	1	100	214	1	100
	Thy-1⁻CD34⁺	2.1	5,250	6.7	0.14	375	1.8	3.8
	Thy-1⁺CD34⁺	0.56	159,000	204	114	44,800	209	117
2	Unsorted	100	1,660	1	100	638	1	100
	Thy-1⁻CD34⁺	2.6	9,150	5.5	14	2,550	4	10
	Thy-1⁺CD34⁺	0.39	80,500	48	19	45,900	72	28
3	Unsorted	100						
	Thy-1⁻CD34⁺	1.5	ND	ND	ND	ND	ND	ND
	Thy-1⁺CD34⁺	0.48						
4	Unsorted	100	174	1	100	68	1	100
	Thy-1⁻CD34⁺	1.4	3,000	17	24	900	13	18
	Thy-1⁺CD34⁺	0.015	112,000	644	97	43,800	644	97

BM cells were sorted on the basis of Thy-1 labeling and CD34 expression, and placed in long-term culture. Long-term cultures were harvested at week 5 or 8, plated in methylcellulose, and scored for colonies on day 14. LTC-IC were assumed to produce an average of four CFU after 5–8 wk in culture (18). Enrichments were calculated by dividing the frequency of CFU in sorted fractions by the frequency for unsorted PI⁻ cells. Recoveries were calculated by multiplying the enrichment by the percent sorted.

and FL cells (not shown) but the majority of CD34⁺ cells from each source were c-*kit*⁺ (Fig. 6, *A*, *D*, and *G*). In all instances when CD34 was plotted against c-*kit*, the c-*kit*^{lo} cells expressed the highest levels of CD34 (data not shown) and Thy-1 expression was found to be restricted to cells that express intermediate to low levels of c-*kit* (Fig. 6, *A*, *D*, and *G*). Thy-1 expression was slightly lower on CB CD34⁺ cells than on comparable BM or FL cells. 5E10-FITC was used in these experiments because detection of c-*kit* expression required sensitive indirect staining. Staining with 5E10-FITC resulted in significantly weaker staining than in comparable stainings in which a biotinylated primary or secondary Ab was used (Fig. 2). The majority of Thy-1⁺CD34⁺ cells were CD71^{lo} in all tissues and Thy-1 expression decreased with increased CD71 expression (Fig. 6, *B*, *E*, and *H*). Similarly, Thy-1 staining was highest on CD38⁻CD34⁺ cells, and decreased with increased CD38 expression (Fig. 6, *C*, *F* and *I*). Rh123 staining and Thy-1 expression on BM CD34⁺ cells were inversely correlated (Fig. 7). Thy-1⁺CD34⁺ cells were Rh123^{dull}, with Rh123 staining increasing as Thy-1 expression decreased. Resolution between Rh123^{dull} and Rh123^{bright} cells was improved by incubating Rh123-stained samples in medium at 37°C to allow dye efflux (compare Fig. 7, *C* and *D*). These FACS® data derived from three-color stainings indicate that Thy-1 has very restricted expression that, within the CD34 compartment, is confined to cells that are phenotypically similar to the most primitive hematopoietic cells.

Phenotypic Analysis of Thy-1^{2+}CD34⁻ Cells in BM and PB. BM and PB cells were analyzed for the coexpression of Thy-1 and various lymphocyte and myeloid markers. Samples were labeled with 5E10, 8G12 (anti-CD34), and various lineage markers (Fig. 8), and Thy-1^{2+}CD34⁻ cells (gate shown in Fig. 2 *A*) were analyzed for the expression of lineage markers. The Thy-1 gate was set high to include only cells that were more brightly stained than control-stained samples (Fig. 2, *A* and *B*). It is possible that, as a result, some weakly stained Thy-1⁺ cells were excluded, however, discrimination between specific and nonspecific staining is difficult in this region. Thy-1^{2+}CD34⁻ cells comprised 0.1–0.3% of low density BM and PB cells, including a small subset of lymphoid cells as judged by their light scatter. As shown in Fig. 8, the majority of Thy-1^{2+}CD34⁻ cells were CD3⁺ and CD4⁺, while a small proportion were CD8⁺ or CD9^{weak}. Cells observed in cytospin preparations of Thy-1^{2+}CD34⁻ cells were small dense lymphocytes (results not shown).

Effect of mAb 5E10 on Cells in Culture. In an attempt to determine whether 5E10 had any functional effects on hematopoietic cells in vitro, 5E10 or control Ab were added at 10 μg/ml to purified BM cells (CD34⁺CD71^{lo}CD45RA^{lo}) plated in Dexter type long-term cultures (2,000 cells/dish) and progenitor assays (400 cells/dish). No significant difference in colony formation was seen between the cultures with (22 colonies/dish) or without added 5E10 Ab (25 colonies/dish). Long-term cultures harvested after 5 wk produced

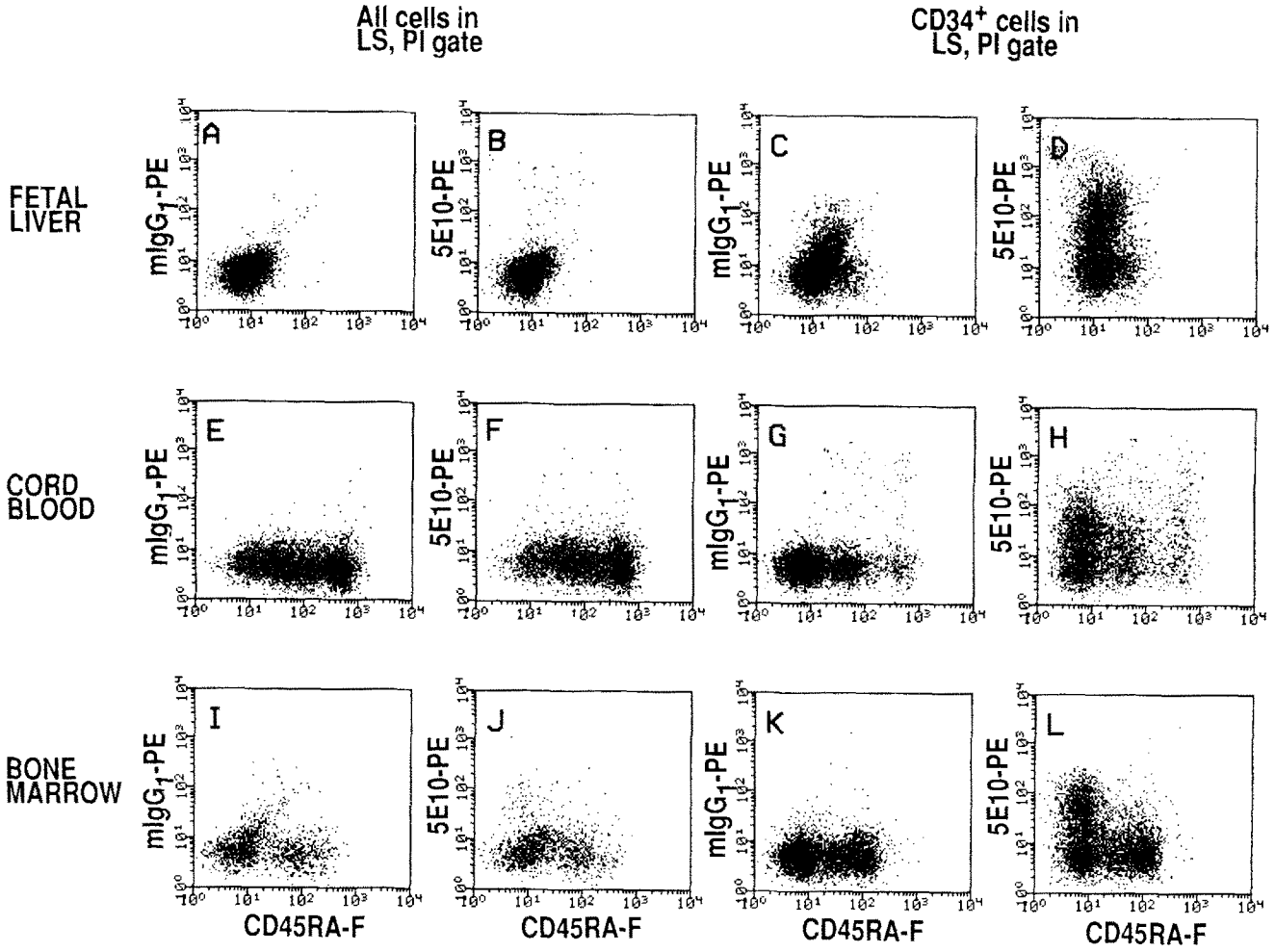

Figure 5. 5E10 staining of $CD34^+$ subpopulations in BM, CB, and FL. Low density mononuclear cells from FL, CB, and BM were stained in parallel with 5E10, anti-CD34, and anti-CD45RA. Gates were set to exclude dead and high side scatter cells as shown in Fig. 1 to reduce nonspecific events, and to restrict analysis to $CD34^+$ cells. 5E10 staining was strongest on $CD34^+CD45RA^-$ cells previously shown to be highly enriched for LTC-IC. Note that 5E10 staining is stronger on FL than CB or BM cells.

172 and 141 colonies/dish for cultures with and without added 5E10, respectively. Week 8 long-term cultures contained 70 LTC-IC/dish for cultures with and without added 5E10. From these experiments it was concluded that, under conditions used, 5E10 did not appear to interfere with the production and/or formation of hematopoietic colonies from primitive hematopoietic cells.

Discussion

In this report we describe the distribution of Thy-1 on human hematopoietic cells. This work was performed with a new mAb, 5E10, which immunoprecipitated a 25–35-kD protein as reported for other anti-Thy-1 antibodies (24, 25), and was formally demonstrated to be directed against Thy-1 by cDNA cloning.

$Thy\text{-}1^+CD34^+$ cells were characterized as being functionally and phenotypically the most primitive cells. This population was highly enriched for LTC-IC measured at weeks 5 and 8 and for HPP-CFC, and depleted of clonogenic cells. FACS® analysis indicated that Thy-1 expression was highest on $CD34^+$ cells that were previously described as being highly enriched for primitive hematopoietic cells (Figs. 5 and 6), in that Thy-1 expression decreased with increased expression of CD45RA, CD38, or CD71 expression (6, 7, 10). Our findings are in agreement with murine studies indicating that, among progenitor cells, Thy-1 expression decreases with commitment and differentiation (26). Thy-1 expression appears to be very low on $CD34^+$ cells, as staining, even with very sensitive techniques, was weak. This weak staining is unlikely to reflect low Ab affinity because 5E10 could be used at relatively low concentrations ($<5\ \mu g/ml$), and certain cell lines, in addition to the rare population of $CD34^-Thy\text{-}1^{2+}$ normal lymphoid cells, were very brightly stained even by directly conjugated Ab.

CB, BM, and FL profiles obtained with 5E10 and CD34 were quite similar except that CD34 and Thy-1 expression appeared higher on some FL cells than on any BM or CB

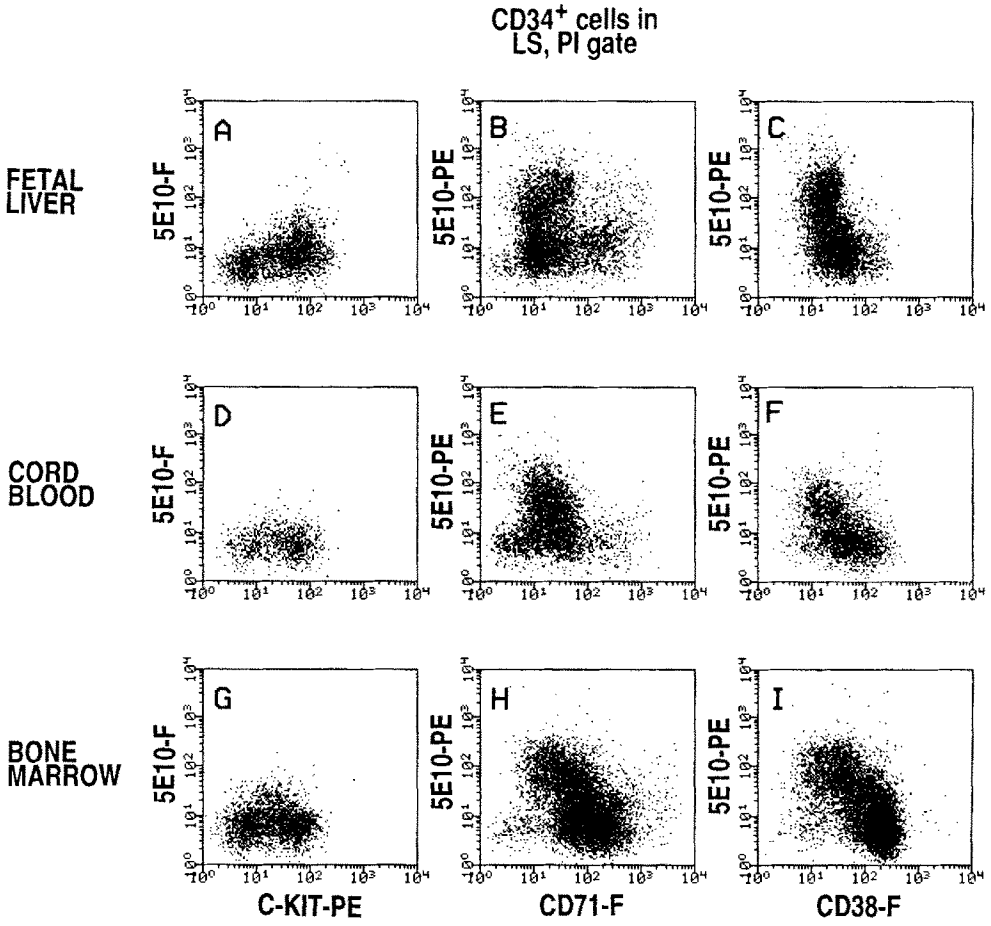

Figure 6. Three-color immunofluorescence staining of CD34+ cells in the light scatter gates (Fig. 1) from BM, CB, and FL with 5E10 and mAbs against the indicated cell surface molecules.

cells, and CD34+ cells in most FL samples examined lacked CD45RA or expressed very little (Fig. 5, *D*, *H*, and *L*, respectively). It is tempting to speculate that this population of Thy-1^{2+}CD34+ cells represents a more primitive population of cells that is lost during ontogeny. Although background staining was greater on FL CD34+ cells than CB or BM CD34+ cells, the increased 5E10 staining is unlikely to be a staining artifact. The higher background may be due to low affinity $F_c\gamma R$ present on FL blast cells (27). Indeed, when FL cells were stained for F_cR (CD16, CD32) a significant proportion (>30%) of FL but <5% of CB or BM CD34+ cells were found to express CD32 and CD16, in agreement with findings by others (27); however, all 5E10+CD34+ cells were F_cR^- (results not shown). The lack of CD45RA expression on the majority of FL CD34+ cells examined coincides with expression of CD45RO (W. Craig and P. M. Lansdorp, manuscript in preparation) and underscores the importance of CD45 isoform expression in the differentiation of hematopoietic cells (7).

Mast cell growth factor (MGF), a ligand for the c-*kit* receptor tyrosine kinase, has powerful synergistic effects with other early-acting growth factors on primitive hematopoietic cells (6, 16), and murine studies indicate that the most primitive hematopoietic cells (those with marrow repopulating ability) are c-*kit*+ (28). We found low levels of c-*kit* expression on human hematopoietic cells that express Thy-1. Because Thy-1 expression appears restricted to phenotypically and functionally the most primitive cells (this report), it thus appears that such cells express low levels of c-*kit*. All CD34+ Rh123^{dull} cells were Thy-1+, with Thy-1 expression being inversely related to Rh123 staining (Fig. 7). This is in contrast to the findings of Baum et al. (4), who found that Thy-1+CD34+ cells could be subdivided using Rh123 staining, however, no data were shown.

The function of BM and CB Thy-1^{2+}CD34- lymphoid cells is unknown. The CD3+CD4+ phenotype suggests a mature cell type, and FACS®-sorted CD34-5E10+ cells indeed appeared to be small lymphocytes in cytospin preparations. The proportion of Thy-1+ lymphocytes increased dramatically upon stimulation of PBL with PHA and IL-2, suggesting that Thy-1 expression is perhaps related to T cell activation (results not shown). To our knowledge, this is the first report of Thy-1+ lymphocytes in normal human BM, although previous reports describe a population of Thy-1+ thymocytes believed to be prothymocytes (25). The only other report of mature Thy-1+ cells described a population of Thy-1+-infiltrating leukocytes in several patients with malignant breast tumors, however, this population was not further characterized (29).

The function of Thy-1 on hematopoietic stem cells is un-

Table 3. *The Percentage of Thy-1$^+$ Cells among Cells within the Indicated Gate/Phenotype*

Sample	PI gate	LS gate	CD34$^+$ cells				
			All	CD71$^-$	CD38$^-$	CD45RA$^-$	c-*kit*$^+$
NBM	2.4	–	25(2.7)	45	80	–	–
	4.2	4.0	20(3.2)	15	32	25	–
	2.5	5.2	41(2.3)	75	75	52	70
	2.5	2.0	26(1.7)	–	–	–	–
	1.0	1.0	22(3.2)	–	–	–	–
	2.0	4.5	27(4.0)	–	87	–	–
	1.8	2.4	21(1.5)	–	–	–	–
	0	0	12(2.5)	–	–	–	–
	2.6	0.6	30(4.6)	–	–	40	50
PB	0.2						
	0.3						
	0.7						
CB	5.8	1.2	54(0.4)	56	45	–	–
	0	0	10(1.0)	–	–	20	–
	1.6	0	25(0.4)	–	–	30	–
	4.5	3.5	40(1.4)	–	–	43	40
FL	3.0	3.0	18(1.0)	30	25	20	70
	7.0	7.0	35(7.4)	50	65	45	60
	1.4	1.3	38(1.3)	65	80	50	30

The reactivity of 5E10 with phenotypically defined mononuclear cells from normal bone marrow (NBM), PB, CB, and FL. Results are expressed as the percent positive cells among the indicated cell fraction (–, not tested). The percent of Thy-1$^+$ cells was determined by subtracting percent positive for the isotype control from the percent of 5E10$^+$ cells. Gates were set as in Fig. 1. The percent of CD34$^+$cells in the PI and light scatter gates of a sample is shown in parentheses.

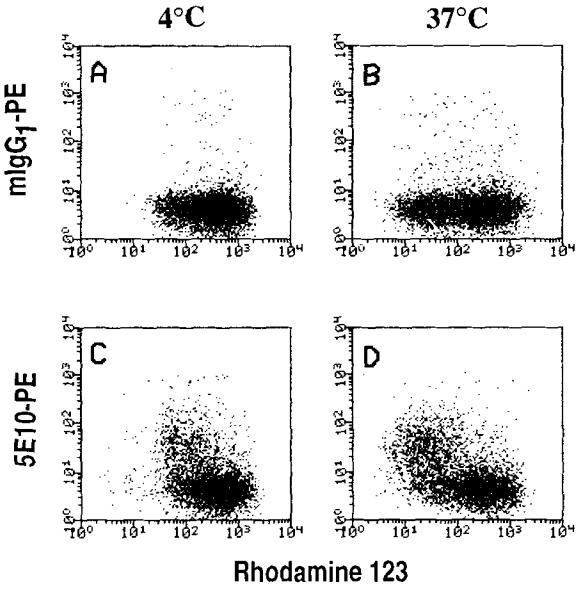

Figure 7. Rh123 staining vs. Thy-1 expression on low side scatter CD34$^+$ adult BM cells. Cells were labeled with 8G12-Cy5, Rh123, and either an irrelevant isotype-matched control Ab (*A* and *B*) or 5E10 (*C* and *D*). After Rh123 staining at 37°C, samples were split and either left on ice at 4°C (*A* and *C*) or incubated an additional 20 min at 37°C (*B* and *D*) to allow dye efflux. Within the CD34 gate, Thy-1 expression (5E10 staining) and Rh123 staining appeared to be reversibly correlated.

known. Rat stem cells express more Thy-1 than primitive hematopoietic cells of murine (30) or human origin, but the fact that Thy-1 expression is conserved on these cells implies an important role for Thy-1 in early hematopoiesis. Recently it was reported that some cells with marrow repopulating ability (presumably stem cells) were Thy-1$^-$ in Thy-1.2 strains of mice, whereas all cells with marrow-repopulating ability in Thy-1.1 strains were Thy-1$^+$ (31). However, in view of the weak expression of Thy-1 on hematopoietic cells, these findings may reflect unusual binding properties of the Abs used.

Thy-1 has been postulated to be involved in cellular recognition (32, 33), adherence (34, 35), and T cell activation (36–39). Some Abs against Thy-1 are able to stimulate intracellular Ca^{2+} release (36) and phosphorylation of cytoplasmic proteins (40), suggesting Thy-1 may be involved in cell activation. Thy-1 is anchored in the membrane by a phos-

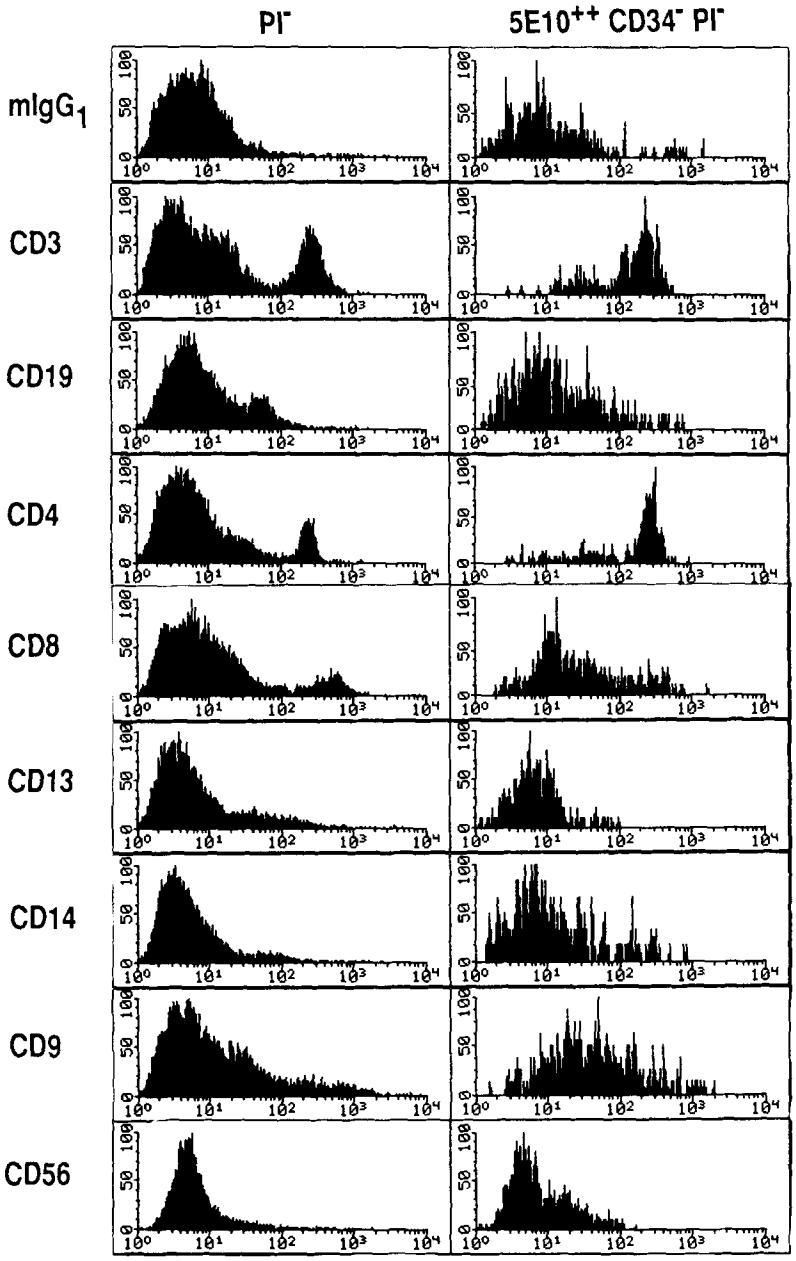

Figure 8. Lineage marker expression on $5E10^{2+}CD34^{-}$ cells. Normal BM cells were labeled with an anti-CD34 Ab, 5E10, and various lineage markers. Histograms on the left are the fluorescence profiles (log scale) for all viable low density mononuclear cells obtained with the indicated marker, whereas histograms on the right represent lineage marker expression on cells in the $5E10^{2+}CD34^{-}$ gate shown in Fig. 2 *A*.

phatidylinositol anchor and has no direct link to the cytoplasm (41); therefore, if it has a role in transmembrane signaling, additional associated proteins would be required. Some murine studies have linked Thy-1 to CD45 (a transmembrane protein with tyrosine phosphatase activity) in cocapping, and crosslinking and immunoprecipitation studies (42, 43). Anti-Thy-1 Abs also coimmunoprecipitate $p56^{lck}$ (44), (a *src*-related tyrosine kinase that can associate with CD45) from murine T cells (45). It is tempting to speculate that on human hematopoietic cells, Thy-1 and CD45RO could be functionally associated, given their coexpression on the most primitive $CD34^{+}$ cells, and the biochemical data physically linking CD45 and Thy-1 in the murine system (42–45).

Thy-1 may be important in stromal adherence, possibly providing a growth inhibitory signal as has been suggested for neuronal Thy-1 (46). Studies on myogenesis (47) and murine thymocytes (35) have indicated that Thy-1 may function as a receptor for a ligand on other cells allowing cells to contact and adhere. Thy-1 may have an analogous role on stem cells, allowing them to recognize and bind to stromal determinants. The lower expression of Thy-1 on circulating $CD34^{+}$ cells from umbilical CB may result from a reduced requirement for adherence and is in agreement with this proposed function of Thy-1.

mAbs against human Thy-1 appear to be attractive tools for stem cell purification techniques because of the very re-

stricted pattern of Thy-1 expression within the human hematopoietic system and the possibility to target such rare cells by immunoaffinity techniques. Anti-Thy-1 Abs have been used previously for the purification of murine (48, 49), rat (50), and recently human fetal BM stem cells (4). Our findings indicate that anti-Thy-1 Abs appear useful for the purification of adult stem cells from human BM as well. Compared with the studies reported by Baum et al. (4), we found a higher percentage of Thy-1$^+$CD34$^+$ cells, but this probably reflects differences in the Abs used. In previous studies anti-Thy-1 Abs have been used to purge marrow for autologous BM transplantation in neuroblastoma patients because the majority of malignant neuronal tumors express very high levels of Thy-1 (24, 51). While such techniques may spare Thy-1$^+$ BM cells because of their much lower Thy-1 expression, improvements in purging techniques could result in the unwanted elimination of Thy-1$^+$ hematopoietic cells, and such techniques should therefore be approached with caution.

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