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Leukemic Burden in Subpopulations of CD34$^+$ Cells Isolated From the Mobilized Peripheral Blood of α-Interferon–Resistant or –Intolerant Patients With Chronic Myeloid Leukemia

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We attempted to determine the frequency of normal hematopoietic stem cells (HSC) and contaminating leukemic cells in mobilized peripheral blood (MPB) collected from chronic myeloid leukemia (CML) patients, intolerant of α-interferon or with interferon-resistant disease. A total of 14 MPB samples, six from patients in chronic phase (CP) and eight from patients in accelerated phase or blast crisis (AP/BC) were studied. Cytogenetic analysis of MPB collected from AP/BC patients showed that 100% of the cells were Ph$^+$, whereas cells from four of five CP MPB were Ph$^-$. By contrast, fluorescence in situ hybridization (FISH) analysis of CP MPB showed a mean frequency of 14.7% Ph$^+$ cells, while AP/BC MPB contained 39.2% Ph$^+$ cells. In an attempt to purify normal HSC, subpopulations of the MPB CD34$^+$ cells were isolated based on expression of the Thy-1 antigen (CDw90). The mean Ph$^+$ cell frequency as determined by FISH within the CD34$^+$Thy-1$^+$ Lin$^-$ and CD34$^+$Thy-1$^-$Lin$^-$ populations from CP patients was 19.2% and 33.9%, respectively. In the AP/BC patients, levels of residual leukemic cells were significantly greater with mean Ph$^+$ cell frequencies of 59.2% and 72.7% for the CD34$^+$Thy-1$^+$Lin$^-$ and CD34$^+$Thy-1$^-$Lin$^-$ fractions, respectively. The frequency of cobblestone area forming cells (CAFC) was used as a means of quantitating the numbers of functional HSC within these cell subpopulations. The mean CAFC frequency was 1 of 19 for the CD34$^+$Thy-1$^+$Lin$^-$ cells as compared with 1 of 133 for the Thy-1$^-$ fraction indicating a higher frequency of primitive progenitor cells in the Thy-1$^+$ subpopulation. CD34$^+$ cell subsets from two patients were also injected into SCID-hu bone assays to determine the in vivo behavior of these cell populations. After 8 weeks, multilineage donor engraftment was observed in these grafts. FISH analysis of the donor cells within the grafts showed that 55.3% and 60.0% of the cells were Ph$^+$. We conclude that unfractionated MPB from this patient population is not leukemia-free and that the CD34$^+$Thy-1$^+$Lin$^-$ cell subpopulation, although predominantly enriched for normal HSC, still contains substantial numbers of residual leukemic cells.

CHRONIC MYELOID LEUKEMIA (CML) is a clonal myeloproliferative malignancy that originates at the level of primitive hematopoietic stem cells.[1,2] In a significant proportion of CML patients, allogeneic bone marrow transplantation (BMT) results in prolonged disease-free survival and is likely curative in some patients for at least a certain period of follow-up.[3-5] Unfortunately, most patients are not suitable for allogeneic BMT due to lack of an appropriate available donor or because of relatively advanced age. McGlave et al[6] recently reported the results of a multicenter study that suggests autologous transplantation can prolong the survival of patients with CML. However, the persistence of leukemic cells within the marrow and peripheral blood of patients posttransplantation was evident in a majority of the survivors.[6] Relapse of these patients may result from the persistence of tumor cells within the patients following intensive myeloablative therapy and/or be the result of tumor cells present in the graft. Support for the latter explanation has been provided using gene marking to demonstrate that CML cells present in bone marrow autografts can contribute to relapse.[7] Therefore, elimination or reduction of the number of leukemic cells within autografts might result in an increase in prolonged disease-free survival following autologous transplantation.

Evidence for the presence of normal and Ph$^+$ hematopoietic stem cells (HSC) in the marrow and peripheral blood of CML patients has been reported previously.[8-15] Two recent reports dealing with the examination of primitive hematopoietic progenitors isolated from the marrow of patients with CML in chronic phase (CP) have indicated the presence of cells containing the Ph chromosome that are not expressing the *bcr-abl* fusion product.[16,17] Such findings may explain the expansion of the leukemic clone at the level of the mature myeloid progenitor without a selective expansion of the primitive CML HSC. Therefore, purification of HSC from CML patients at a stage before expression of the *bcr-abl* transcript could lead to the isolation of autografts containing primarily normal HSC. Verfaillie et al[14] have reported the presence of exclusively normal HSC within the HLA-DR low/negative (DR$^-$) subfraction of CD34$^+$ from early chronic phase CML bone marrow. Leemhuis et al[15] using a similar cell population from chronic phase CML marrow have also identified normal HSC, but at a lower frequency. Recently, Kirk et al,[18] using fluorescence in situ hybridization (FISH) to detect the Ph chromosome, have reported the enrichment of normal HSC within the CD34$^+$DR$^-$, but not in the CD34$^+$CD38$^-$ population of cells isolated from chronic phase CML patients. Together these data indicate that the levels of Ph$^+$ cells within the CD34$^+$DR$^-$ population are reduced compared with the CD34$^+$DR$^+$ population, but that these purified populations still contain considerable numbers of leukemia cells.

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A number of groups have reported the presence of human HSC within subfractions of $CD34^+$ cells (reviewed in Spangrude.[19,20-22]) We have shown previously that the Thy-1 surface marker subsets the $CD34^+$ fraction of fetal BM, adult BM, and mobilized peripheral blood (MPB) and the isolated Thy-1^+ subset is highly enriched for HSC.[23,24] Purification of $CD34^+$Thy-1^+Lin^- HSC from the MPB of multiple myeloma patients has been shown to reduce the level of contaminating tumor below the level of detection.[25] We hoped to determine if purification of these long-term repopulating HSC from the more mature components of the graft in α-interferon resistant or intolerant CML patients would result in a leukemia-depleted HSC graft.

Differential mobilization of normal HSC and tumor cells has also been used to create presumably leukemia-free autografts. Carella et al[26] have reported the preferential mobilization of normal HSC rather than Ph^+ HSC after intensive chemotherapy of interferon-resistant CML patients with idarubicin, etoposide, and cytosine arabinoside (ICE) followed by granulocyte colony-stimulating factor (G-CSF). Ph^- leukapheresis products, as determined by cytogenetic analysis, were collected in 60% of patients treated in chronic phase and 20% to 30% of patients in blast crisis (BC) or accelerated phase (AP). In addition, Kantarjian et al[27] have shown that following intensive chemotherapy of 38 patients with CML leukapheresis products from 28% of patients were free of leukemia cells. Their report confirmed that the peripheral blood of these patients contains fewer leukemia cells than the marrow. Talpaz et al[28] have presented data that suggests the outcome of transplants using these autografts is in part dependent on the percentage of Ph^+ and Ph^- cells within the autograft. We hypothesized that the combination of preferential mobilization of nonleukemic cells during recovery from intensive chemotherapy of CML patients and purification of normal HSC by means of monoclonal antibody (MoAb) labeling and fluorescence-activated cell sorter (FACS) would lead to a further reduction in the leukemic cell level and permit the isolation of a leukemia-depleted HSC graft.

MATERIALS AND METHODS

Patient population and samples. Twelve cytogenetically and morphologically confirmed CML patients in CP, AP, or BC were the subject of this study. Informed consent, approved by the Human Subjects Committee at participating centers, was obtained before the performance of these studies. Patients used for this study were α-interferon intolerant or resistant (defined as $<10\%$ cytogenetic response at 6 months and $<66\%$ cytogenetic response at 12 months), between the ages of 15 and 60, and had no allogeneic donor available. BM aspirates from the posterior iliac crest were obtained before the administration of chemotherapy and G-CSF. Patients were placed on a mobilization protocol, approved by the Human Subjects Committee at participating centers, as previously described by Carella et al.[29] Briefly, patients were mobilized with ICE chemotherapy consisting of idarubicin at 8 mg/m^2 on days 1 to 5, arabinosylcytosine at 800 mg/m^2 in 2-hour infusions on days 1 to 5, and etoposide at 150 mg/m^2 on days 1 to 3. On day 8 postchemotherapy, subcutaneous administration of G-CSF at 5 μg/kg/d was begun and continued until a total neutrophil count of greater than 1×10^9/L was reached for 3 consecutive days. Leukapheresis was initiated when the white blood cell (WBC) count was greater than 0.5×10^9 cells/L and was continued until $>5 \times 10^8$ mononuclear cells/kg were collected. Either a Cobe Spectra or a Fenwall System (Cobe Laboratories Inc, Lakewood, CO or Baxter Healthcare Corp, Fenwall Division, Deerfield, IL) was used for leukapheresis.

Sample shipment and processing. Fresh leukapheresis samples were shipped (World Courier, Jamaica, NY) to the Systemix cell processing facility in Palo Alto, CA. Upon receipt, 0.05 to 5×10^6 mononuclear cells were removed for slide preparation ultimately to be used for FISH analysis of the number of Ph^+ cells. Mononuclear cells were separated from the remainder of the sample by density centrifugation using Ficoll-Hypaque (Pharmacia, Piscataway, NJ). $CD34^+$ cells were first enriched using a magnetic bead-mediated technique with release of $CD34^+$ cells by the glycoprotease enzyme.[30] MPB cells were resuspended in staining buffer (SB = HBSS, 2% fetal bovine serum (FBS; Hyclone Labs, Logan, UT), 10 mmol/L HEPES) at 5×10^7 cells/mL. QBEnd10 anti-CD34 MoAb (Immunotech, Westbrook, ME) was added at a 1/100 dilution, and cells were incubated for 30 minutes on ice. Cells were then washed in staining buffer with an FBS underlay, and resuspended at 4×10^7 cells/mL. An equal volume of washed Dynal sheep antimouse IgG_1Fc magnetic beads (Dynal, Oslo, Norway) was added at a 1:1 bead to cell ratio, to give a final cell concentration of 2×10^7 cells/mL. After a 30-minute incubation period on ice (with gentle inversion), the tube was placed against a Dynal magnet for 2 minutes. Following two washes with staining buffer, 20 μL of O-sialoglycoprotein endopeptidase (CLE100; Accurate Chemical, Westbury, NY) plus 180 μL of RPMI (JRH Biosciences, Woodland, CA)/20% FBS were added and the beads incubated for 30 minutes at 37°C to release $CD34^+$ cells from the beads. The beads were then washed three times with SB to maximize recovery. The $CD34^+$ cells were then maintained overnight at 4°C before staining for flow cytometry or cryopreserved under liquid nitrogen in RPMI supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO) at a cell concentration of 1×10^7 cells/mL.

Cytogenetics. Cytogenetic analysis of BM aspirates and MPB leukapheresis products were performed using standard metaphase cytogenetic techniques in the cytogenetic laboratories in Genova, Italy; Paris, France; Duarte, CA; and Palo Alto, CA.

Slide preparation of unfractionated samples. Leukapheresis samples were centrifuged in a table-top centrifuge for 8 minutes at 900 rpm (200*g*), 22°C to pellet the cells. Red blood cells were removed by resuspending the cell pellet in 0.84% ammonium chloride lysis solution for 5 minutes at 4°C. Mononuclear cells were isolated by centrifugation and washed once in RPMI supplemented with 10% FBS. Slides were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA). Approximately 1 to 5×10^4 cells in RPMI/10% FBS were added to each sample chamber and centrifuged for 3 minutes at 450 rpm. Slides were removed from the cytocentrifuge and air-dried. The slides were fixed with methanol:acetic acid (3:1) for 15 minutes and allowed to air-dry. Slides were stored at −80°C until use.

Slide preparation of purified samples. Flow cytometry-purified cells were pelleted in 15 mL screw-capped tubes using a table-top centrifuge and washed once with RPMI containing 10 U/mL of heparin (ICN Biomedicals Inc, Aurora, OH). The cells were resuspended in 100 to 200 μL of RPMI/heparin for each 20,000 cells. A PAP pen (Research Products International, Mt. Prospect, IL) was used to mark a rectangle on silane-treated slides, and 100 to 200 μL of cell suspension was spread throughout the rectangle. Slides were incubated for 30 to 45 minutes at room temperature (RT) to allow the cells to settle and attach to the slides. Excess liquid was removed by tipping the slides sideways and the slides were air-dried. The slides were fixed with methanol:acetic acid (3:1) for 15 minutes and allowed to air-dry. Slides were stored at −80°C until use.

FISH. On the day of hybridization, slides were thawed at RT, refixed with methanol:acetic acid (3:1), and air-dried. As a pretreat-

ment, the slides were incubated in 2× SSC (3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.0) for 30 minutes at 37°C, followed by dehydration in a series containing ethanol at a concentration of 70%, 90%, and 100% at RT. In some cases, the slides were treated by pepsin (Sigma, St Louis, MO) to improve probe penetration.[31] Slides were denatured in 70% formamide/2× SSC for 2 minutes at 72°C followed by dehydration in a series containing ethanol at a concentration of 70%, 90%, and 100% on ice. The CML translocation probe (Oncor, Gaithersburg, MD) consisted of a mixture of digoxigenin-labelled probes for the *bcr* locus and biotin-labelled probes for the c-*abl* locus. Hybridization was performed according to manufacturer's instruction with minor modifications. The probe was prewarmed for 5 minutes at 37°C before addition to the prewarmed slides. Slides were coverslipped, sealed, and incubated overnight at 37°C in a humidified chamber. The next day, the coverslips were removed, and the slides were washed three times in 50% formamide/2× SSC for 5 minutes each at 43°C followed by two washes in 2× SSC for 10 minutes each at 37°C. A final wash in 0.1× SSC for 5 minutes at 37°C was performed. Before detection, the slides were blocked by incubation in detection staining buffer (DSB = 4× SSC/0.05% Tween-20/10% normal rabbit serum [NRS; Vector Laboratories, Burlingame, CA]) for 30 minutes at 37°C. Detection of the bound probe was performed using a two-step immunodetection system. For the first step, a 1:100 dilution of mouse antidigoxigenin MoAb (Boehringer Mannheim, Indianapolis, IN) and a 1:100 dilution of fluorescein isothiocyanate (FITC) conjugated streptavidin (Vector Laboratories) was made in DSB. Slides were incubated in this mixture for 45 minutes at 37°C followed by three washes in 4× SSC/0.05% Tween for 5 minutes each at RT. For the second step, a 1:50 dilution of rhodamine-conjugated sheep antimouse $F(ab')_2$ antibody (Boehringer Mannheim) and a 1:100 dilution of FITC-conjugated goat antistreptavidin antibody (Vector Laboratories) was made in DSB. Slides were incubated in this mixture for 45 minutes at 37°C followed by three washes in 4× SSC/0.05% Tween for 5 minutes each at RT. The slides were counterstained with 0.3 μg/mL 4′,6-diamino-2-phenylindole (DAPI; Sigma) in 4× SSC/0.05% Tween for 5 minutes at RT and mounted in Vectashield mounting media (Vector Laboratories). Slides were analyzed with a Nikon photomicroscope equipped with epifluorescence and 100×/1.25 oil Plan-Neofluoar objectives. Cells were visualized using a triple-band pass filter (Chroma, Brattlebro, VT). Images were taken using a C·Imaging 1280 analysis system (Compix Inc, Mars, PA) using a CCD camera (Photometrics, Tucson, AZ), automated stage and filter wheel (LUDL, Hawthorne, NY), and SIMPLE software (Compix Inc). Individual images were taken through DAPI, FITC, and Rhodamine band-pass filters (Chroma) in combination with neutral density filters, pseudocolored blue, green, and red, respectively, and layered on top of one another to give the final color image. The image was digitally stored in tagged image file format (TIFF) and was imported into Microsoft Imager (Microsoft, Redmond, WA) for adjustment of the brightness and contrast of the image such that the final print image matched the original video image. Print images were made from negatives obtained by placing the digital image onto a film negative using a film recorder. All analyses were performed using cells with good nuclear morphology and complete, nonoverlapping nuclei. A cell was scored Ph^- if two distinct red and two distinct green signals were visible within a nucleus. A cell was scored Ph^+ if it contained one distinct red signal, one distinct green signal, and one distinct yellow signal representing the coincidence of a red and green signal. In addition, a cell was scored Ph^+ if it contained two distinct red signals and two distinct green signals with one red signal and one green signal in close juxtaposition. All other cells that contained fewer signals were not scored as Ph^+ or Ph^-, but were used to calculate the hybridization frequency, which ranged from 90% to 95% for this study. Any cells that contained additional signals consistent with the presence of more than one Ph^+ chromosome were scored as Ph^+. Hybridization and analysis of several normal blood samples as controls resulted in a Ph^+ frequency between 1.0% and 1.4%, which represents the background associated with the FISH technique for this application.

Immunostaining for flow cytometry. Cells were resuspended in phosphate-buffered saline (PBS) 0.02% gamimmune (Miles Inc, Elkhart, IN) for 10 minutes on ice and then stained with reagents prepared by Systemix' Product Development and Operations divisions: Sulforhodamine 101 conjugated anti-CD34 $F(ab')_2$, 2.5 μg/mL (Tuk3 from A. Ziegler, University of Berlin, Berlin, Germany), biotinylated anti-Thy-1 (GM201 from Dr W. Rettig, Ludwig Cancer Institute, New York, NY), fluoresceinated anti-CD14, 1 μg/ml (Tuk4 from A. Ziegler), fluoresceinated anti-CD15, 1 μg/mL (Tuk9 from A. Ziegler). After 20 minutes on ice, cells were removed as a control, and streptavidin (ProZyme Inc, El Cerrito, CA) was added to a final concentration of 100 μg/mL for an additional 20 minutes on ice. After washing the cells in SB, biotinylated R-phycoerythrin (PE; Martek BioScience Inc, Columbia, MD) was added at 10 μg/mL to both tubes and cells incubated for a further 20 minutes on ice. After a final wash in SB, cells were resuspended in 1 μg/mL propidium iodide (Boehringer Mannheim) and sorted on the FACSTAR Plus cell sorter (Becton Dickinson) equipped with dual argon ion lasers, the primary emitting at 488 nm and a dye laser (Rhodamine 6G) emitting at 600 nm (Coherent Innova 90, Santa Clara, CA). MPB $CD34^+Lin^-$ (Lin^- = CD14 and CD15 negative populations) cells were divided into Thy-1^+ and Thy-1^- cell subsets, setting the Thy-1^+ gate so as to contain <1% of $CD34^+$ cells stained with the primary control antibodies. Cell populations sorted were from the lymphoblastoid region (R1) after removal of cells with high propidium iodide uptake and electronically gating out Lin^+ cells (R2).

CAFC assay. The CAFC assay was performed as previously reported.[24] Briefly, SyS1 mouse BM stromal cells were plated in Whitlock-Witte Media[32] in wells of polystyrene flat-bottom 96-well plates (Corning, Corning, NY), and allowed to form a confluent adherent layer. After 1 to 2 weeks, sorted cells were plated onto confluent SyS1 cells at limiting dilution (100 to 1 cells/well, 24 wells per concentration) in long-term culture media (1:1 IMDM/RPMI; JRH Biosciences) containing 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate (JRH Biosciences) and 10^{-6} mol/L 2-mercaptoethanol (Sigma), in the presence of purified interleukin-6 (IL-6) at 10 ng/mL and leukemia inhibitory factor (LIF) at 50 ng/mL, provided by Sandoz Inc (Basel, Switzerland). Cultures were replenished weekly with medium containing the same growth factors. Cobblestone areas consisting of >50 blast-like cells (uniform refractal agranular cells in close association) were enumerated after 5 weeks of incubation. The frequency of CAFC was calculated by the cell number at which 37% of the wells show no growth of cobblestone areas, using regression analysis, with 95% statistical precision.[33]

Severe combined immunodeficient (SCID) hu bone assay. Immunodeficient C.B-17 scid/scid mice were used as recipients of human fetal tissues. Fetal tissues were obtained with informed consent, according to state and federal regulations. Mice were bred under specific pathogen-free conditions in the Systemix animal facility and were transplanted at 6 to 8 weeks of age with human fetal bone fragments. The grafts were allowed to vascularize and establish in the murine surrogate host for 8 weeks before being used. Mice received a single dose (350 rads) of whole body irradiation from a ^{137}Cesium source (JL Shepherd and Associates, San Fernando, CA) immediately before the injection of HLA-mismatched donor cell populations. Sorted cells were injected directly into the bone grafts in a 10-μL volume, using a Hamilton syringe. The bone grafts were removed 8 weeks postcell injection, and the cellular contents were harvested by flushing with PBS/0.2% bovine serum albumin (BSA;

Table 1. Philadelphia-Chromosome Positive Cell Frequency in CML Patient Samples

		% Ph^+ Cells (Ph^+ cells/total cells)*		
		Cytogenetics		FISH
Disease Stage	Sample	PreBM†	MPB‡	MPB
Chronic phase	1	100	0.0	ND
	2	100	30.0	16.1 (15/93)
	3	100	0.0	1.2 (1/85)
	4	100	ND	18.5 (17/92)
	5	100	0.0	23.0 (35/152)
	6	100	0.0	14.9 (15/101)
	Mean	100	6.0	14.7
Accelerated phase or blast crisis	1	100	100	35.0 (14/40)
	2	100	100	7.1 (2/28)
	3	100	100	31.0 (22/71)
	4	100	100	52.2 (48/92)
	5	ND	ND	62.1 (36/58)
	6	100	ND	39.1 (25/64)
	7	100	ND	40.0 (40/100)
	8	100	ND	46.7 (35/75)
	Mean	100	100	39.2

Abbreviation: ND, not determined.

• Percentage of Ph^+ cells was determined by dividing the number of Ph^+ cells by the total number of cells analyzed.

† Number of metaphases varied in each sample, but all detectable metaphases were Ph^+.

‡ A minimum of 20 metaphase spreads were analyzed for each sample.

Sigma), pelleted by centrifugation, and resuspended for 5 minutes in PBS containing 0.84% ammonium chloride to lyse the red blood cells. Cells were then washed twice in PBS-BSA, adjusted to 2 to 5×10^5 cells/mL and immunostained with PE- or FITC-conjugated MoAbs recognizing HLA allotypes in combination with MoAbs to CD19, CD33, CD34 (Becton Dickinson), or irrelevant isotype controls. Flow cytometric analysis of donor cells were performed on a FACScan as described by Chen et al.[34] A positive graft contained >1% cells expressing the donor HLA marker. Ten percent of the cells from each positive graft from a single experiment were pooled and sorted for donor-positive HLA using a FACSTAR Plus cell sorter (Becton Dickinson).[34]

RESULTS

Cytogenetic and FISH analysis of CML patient samples. BM aspirates and MPB specimens from 12 CML patients were analyzed by standard metaphase cytogenetics for the presence of the Ph chromosome. The results from six samples obtained from patients in chronic phase and eight samples from patients in AP or BC are summarized in Table 1. Because the findings were similar for samples from patients in AP or BC, samples from either stage of disease are grouped together. A consistent finding of 100% Philadelphia chromosome positive (Ph^+) cells in bone marrow aspirates (preBM) was found in samples from patients in both CP and AP/BC before high dose chemotherapy. Analysis of the percentage of Ph^+ cells within the MPB collects showed a distinct difference in the levels of cytogenetically detectable leukemia cells between the two patient populations. Samples from patients in CP contained no, or a minor population of, detectable Ph^+ cells with a mean of 6%, while samples from patients in AP/BC were 100% Ph^+. Only one MPB sample from a CP patient contained a considerable number of Ph^+ cells (30%), while the remaining four samples were negative for Ph^+ cells.

These results reflect the level of leukemia cells within the dividing cell population of these patient samples. To further analyze the extent of leukemia cell contamination within the interphase cell population of the MPB samples, FISH analysis was performed using probes for the *bcr* and *c-abl* loci. The results of FISH analysis on MPB samples also show a distinct difference in the levels of Ph^+ cells detected within the two patient populations and are summarized in Table 1. The mean level of Ph^+ cells in the MPB samples from patients in CP and AP/BC were 14.7% and 39.2%, respectively. T-test analysis of the original data indicated a statistical difference between the results for the two patient populations ($P = .005$). Several samples from patients in CP were negative by cytogenetic analysis of metaphase spreads, but contained from 14.9% to 23.0% Ph^+ cells by interphase FISH analysis. In one sample from a CP patient (Table 1; chronic phase sample #3), a 1.2% Ph^+ frequency was found. This frequency of Ph^+ cells is within the background of the assay and cannot be considered positive by FISH. The frequency of Ph^+ cells within samples from patients in AP/BC was 100% by metaphase cytogenetics, but ranged from a low of 7.1% to a high of 62.1% as measured by interphase FISH.

Flow cytometric isolation and in vitro activity of subpopulations of $CD34^+Lin^-$ cells from the MPB of CML patients. The $CD34^+Lin^-$ cell population in the MPB of CML patients was subdivided using the Thy-1 cell surface marker into Thy-1-positive ($Thy\text{-}1^+$) and Thy-1-negative ($Thy\text{-}1^-$) subpopulations. Figure 1A through C shows the gating scheme used during the purification of the subpopulations from a CP patient sample. Approximately 10% of the purified sample was used for reanalysis to determine sort purity based on the original gating scheme (Fig 1D and E). The mean purity of the $CD34^+Thy\text{-}1^+Lin^-$ cell populations was 83% with a range of 74% to 92%, while the mean purity for the $CD34^+Thy\text{-}1^-Lin^-$ population was 73% and a range of 54% to 89%. The lower purity of the $CD34^+Thy\text{-}1^-Lin^-$ populations was primarily due to an increase in the number of CD34-negative cells within this subpopulation (data not shown). The purity reported for each cell population reflects the minimum purity, based on reanalysis using the original sort gates.

To determine the primitive progenitor frequency in the purified subpopulations, a proportion of the isolated cells were assayed for cobblestone area forming cells (CAFC). The results from the $Thy\text{-}1^+$ and $Thy\text{-}1^-$ subpopulations of $CD34^+Lin^-$ cells purified from the MPB of CML patients are summarized in Table 2. In each sample the $Thy\text{-}1^+$ subpopulation contained the highest frequency of CAFC ranging from 1 of 7 to 1 of 126 with a mean of 1 of 19. The $Thy\text{-}1^-$ subpopulation still contained considerable CAFC activity with a mean frequency of 1 of 133.

FISH analysis of the Ph^+ frequency within the purified $CD34^+Lin^-$ subpopulations. To determine the levels of

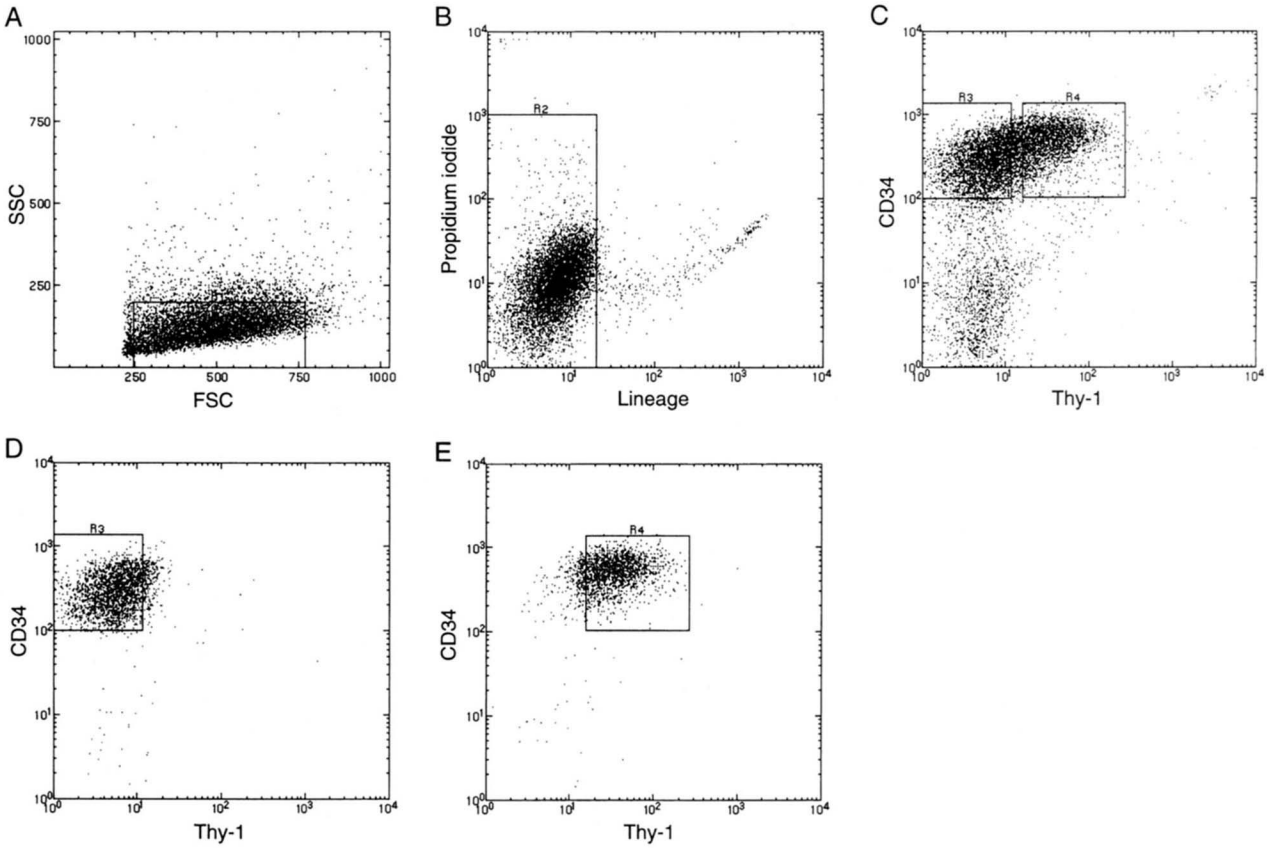

Fig 1. FACS sorting of CD34 and Thy-1 expressing cells from the MPB of CML patients. The MPB samples were ficolled and positively selected resulting in >75% purity of $CD34^+$ cells before sorting. Cell populations sorted were from the lymphoblastoid region (A; region R1) after removal of cells with high propidium iodide uptake and electronically gating-out Lin^+ cells (B; region R2). (C) $CD34^+Thy\text{-}1/Lin^-$ sort. (D) and (E) are reanalysis of $Thy\text{-}1^-$ and $Thy\text{-}1^+$ cell populations, respectively. R3 = $CD34^+Thy\text{-}1^-Lin^-$ and R4 = $CD34^+Thy\text{-}1^+Lin^-$. Purities from the sorts ranged from 54% to 92%, with mean purities of 73% and 83% for the $CD34^+Thy\text{-}1^-Lin^-$ and $CD34^+Thy\text{-}1^+Lin^-$ cell populations, respectively.

Table 2. CAFC Frequencies of Isolated $CD34^+Lin^-$ Subpopulations

Disease Stage	Sample	$Thy\text{-}1^+$	$Thy\text{-}1^-$
CP	1	1/80	1/218
	2	1/14	1/81
	3	ND	ND
	4	1/38	<1/10,000
	5	1/31	ND
	6	1/15	1/29
AP or BC	1	1/126	1/357
	2	1/7	ND
	3	ND	ND
	4	ND	ND
	5	ND	ND
	6	1/22	1/575
	7	1/16	1/461
	8	1/16	1/507
	Mean	~1/19	~1/133

Estimate of the fraction of cells in the input population that give rise to cobblestone areas.

leukemic cells within the $CD34^+Lin^-$ subpopulations, FISH analysis was performed on the purified cells. Table 3 summarizes the results for the $Thy\text{-}1^+$ and $Thy\text{-}1^-$ subpopulations of the $CD34^+Lin^-$ cells isolated from CP patient samples. The mean frequency of Ph^+ cells in the $Thy\text{-}1^+$ and $Thy\text{-}1^-$ subpopulations was 19.2% and 33.9%, respectively. In the overwhelming majority of samples (samples 1 to 5, Table 3), the frequency of Ph^+ cells within the $Thy\text{-}1^+$ subpopulation was lower than in the $Thy\text{-}1^-$ subpopulation. The Ph^+ cell frequency range was from 6.5% to 33.0% for the $Thy\text{-}1^+$ subpopulation and from 20.6% to 48.8% for the $Thy\text{-}1^-$ subpopulation.

FISH analysis of the subpopulations of $CD34^+Lin^-$ cells from CML patients in AP/BC are also summarized in Table 3. The mean frequency of Ph^+ cells within the $Thy\text{-}1^+$ and $Thy\text{-}1^-$ subpopulations was 59.2% and 72.7%, respectively. In most AP/BC patient samples (samples 1 to 7, Table 3), the level of Ph^+ cells was greater in the $Thy\text{-}1^-$ subpopulation compared with the $Thy\text{-}1^+$. The frequency of Ph^+ cells ranged from 28.0% to 78.0% for the $Thy\text{-}1^+$ subpopulation and ranged from 48.7% to 88.0% for the $Thy\text{-}1^-$ subpopulation.

Statistical analysis of the data in Table 3 indicates that

Table 3. Ph^+ Cell Frequency in $CD34^+Lin^-$ Subpopulations From CML Patient Samples

Disease Stage	Sample	% Ph^+ Cells (Ph^+ cells/total cells)* Thy-1^+	Thy-1^-	
CP	1	9.8 (5/51)	20.6 (27/131)	
	2	21.9 (7/32)	47.3 (35/74)	
	3	6.5 (3/46)	ND	
	4	28.1 (27/96)	48.8 (61/125)	
	5	15.7 (25/159)	23.7 (9/38)	
	6	33.0 (33/100)	29.0 (29/100)	
	Mean	19.2	33.9	(P = .034)
AP or BC	1	46.6 (54/116)	ND	
	2	28.0 (14/50)	ND	
	3	77.7 (80/113)	85.1 (86/101)	
	4	75.2 (76/101)	86.4 (89/103)	
	5	76.0 (76/100)	ND	
	6	78.0 (78/100)	88.0 (52/59)	
	7	32.8 (20/61)	48.7 (38/78)	
	8	59.0 (46/78)	55.3 (42/76)	
	Mean	59.2	72.7	(P = .133)
		(P = .0005)	(P = .0029)	

* Percentage of Ph^+ cells was determined by dividing the number of Ph^+ cells by the total number of cells analyzed.

these findings are consistent with a significant difference in the levels of leukemic cells observed within the purified $CD34^+Lin^-$ subpopulations from patients in CP compared with patients in AP/BC. The difference in Ph^+ cell frequency between the CP and AP/BC patient samples is statistically significant for the Thy-1^+ and Thy-1^- subpopulations (P = .0005 and P = .0029, respectively). In addition, the difference in Ph^+ cell frequency between the Thy-1^+ and Thy-1^- subpopulations from CP patient samples is statistically significant (P = .034). In contrast, the difference in Ph^+ cell frequency between the Thy-1^+ and Thy-1^- subpopulations from AP/BC patient samples is not statistically significant (P = .133).

Engraftment of $CD34^+$ subpopulations from CML patient samples in the SCID-hu bone model. The ability of purified populations from the MPB of CML patients to repopulate marrow was tested in the SCID-hu bone assay. In two separate experiments, the $CD34^+$ cells from a CP patient or the Thy-1^+ subpopulation of $CD34^+Lin^-$ cells from an AP patient were injected into human fetal bone implanted in SCID mice. Engraftment of the injected cells after 8 weeks was demonstrated in both experiments by the presence of donor specific HLA-positive cells (Fig 2A). Table 4 summarizes the engraftment results for the two experiments. In experiment one, a majority of the grafts (5 of 8) injected with at least 500,000 cells contained significant levels of donor cells 8 or 9 weeks after injection. In contrast, none of the 10 grafts injected with only 100,000 $CD34^+$ cells contained detectable levels of donor cells. In the second experiment, most grafts (6 of 8) contained significant levels of donor cells 8 weeks after injection, even when as few as 50,000 cells were injected into the graft (4 of 6). To determine the levels of leukemic cells within the grafts, donor cells were isolated by flow cytometry and analyzed by FISH for the presence of Ph^+ cells. The results for both experiments are also summarized in Table 4. Ph^+ cells were detected in both samples indicating that primitive CML progenitor cells within the injected cell population will engraft in the SCID-hu bone assay. In addition, the level of Ph^+ cells was considerably less than 100%, indicating that the normal stem cells within the 50,000 injected cells were also able to engraft and proliferate.

To better understand the nature of marrow repopulation and engraftment of the injected cells, analysis of the donor-derived cells obtained from the SCID-hu mice was performed. The results of flow cytometry analysis using antibodies to the donor-specific HLA and antibodies to CD19, CD33, and CD34 (Fig 2B through D) are summarized in Table 5. Of the donor-derived cells from the six positive grafts from experiment 2, 3.7% were donor-derived $CD19^+$ B cells, 82.3% $CD33^+$ myeloid cells, and 29.3% $CD34^+$ cells. Results of analysis of the host cells are also shown in Table 5. There was an unusually high percentage of $CD33^+$ myeloid cells present within the donor populations compared with the host populations. The mean $CD33^+$ frequency in the donor population was 82.3%, while the frequency was 7.3% in the host population. In addition, the mean $CD19^+$ frequency was 74.9% in the host and 3.7% in the donor populations. Because the levels of $CD19^+$ cells are above 1% for most grafts analyzed, the presence of $CD19^+$ cells in the donor population does signify multilineage engraftment.

DISCUSSION

The current study was initiated to determine the level of normal HSC within subpopulations of $CD34^+Lin^-$ cells isolated from the MPB of CML patients. These studies were directed towards ultimately identifying a leukemia-free autograft for CML patients. Detection of leukemic cells was performed using a two-color FISH analysis of patient samples and compared with results found by standard cytogenetics. The levels of leukemic cells within the HSC population of $CD34^+Thy\text{-}1^+Lin^-$ cells were analyzed and the marrow repopulating ability of this population in the SCID-hu bone model was assessed.

The presence of a Ph chromosome is diagnostic for CML. Unfortunately, cytogenetic analysis only measures the Ph chromosome content in actively dividing cell populations, ignoring the vast majority of quiescent cells in interphase that may be Ph^+. In addition, reverse transcriptase-polymerase chain reaction (RT-PCR) amplification has been used to test for the presence of leukemic cells within patient samples, but several recent reports have indicated that the fusion gene may have variable levels of transcription in primitive hematopoietic progenitor cell populations.[16,17] The use of FISH to detect the presence of the Ph chromosome has been reported by several groups.[35,36] FISH analysis of the Ph chromosome can be performed on metaphase or interphase cells and does not require the expression of the fusion product. In addition, quantitation of the positive cell frequency is more precise than quantitation of PCR amplification products.

Data on the frequency of leukemic cells in the MPB samples indicate that FISH analysis is a more sensitive means of detecting Ph^+ cells than cytogenetic analysis. Four sam-

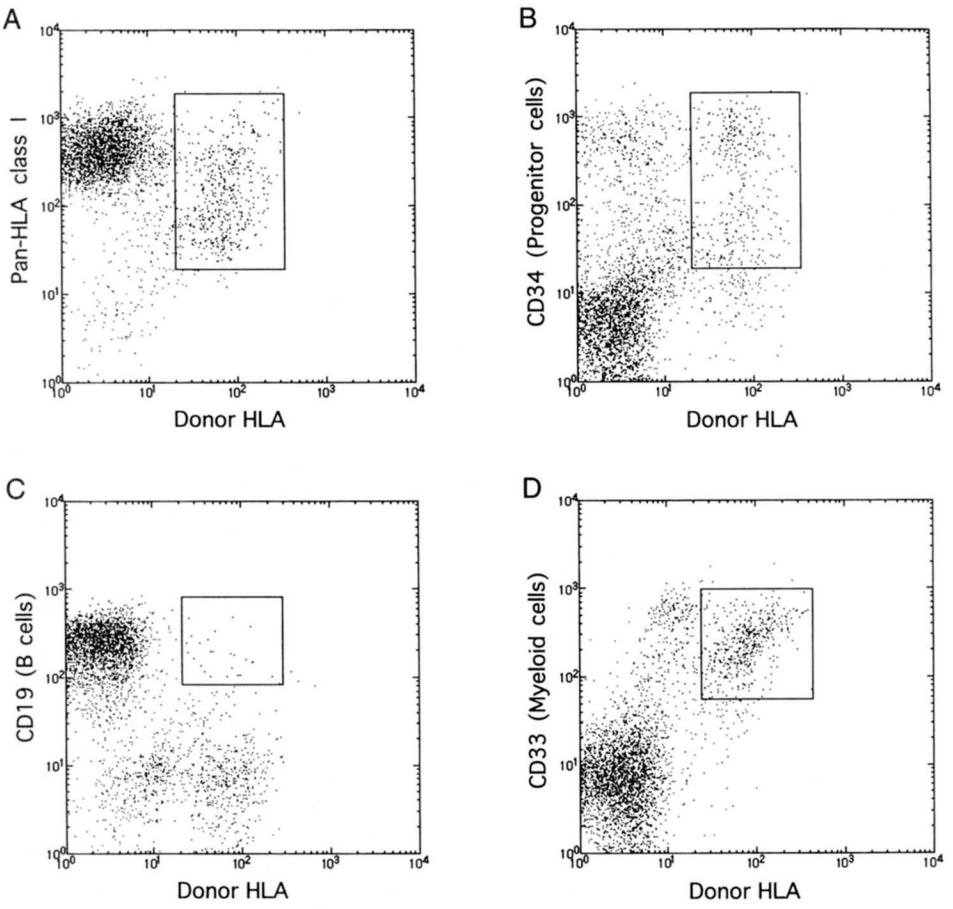

Fig 2. Engraftment of CD34$^+$ Thy-1$^+$Lin$^-$ cells from the MPB of a CML patient in the SCID-hu bone model. Positive engraftment of >1% donor human cells (A), donor CD34$^+$ progenitor cells (B), donor CD19$^+$ B lymphocytes (C), and donor CD33$^+$ myeloid cells (D) were observed.

ples from CP patients were Ph$^-$ by cytogenetics, while the sixth contained 30% Ph$^+$ cells. However, FISH analysis showed levels of leukemic cell contamination between 14.9% and 23.0%. In only one of the samples was the level of Ph$^+$ cells below the background, indicating a Ph$^+$ frequency that is <1.5%. In addition, all samples tested from patients in AP or BC were 100% Ph$^+$ by cytogenetics, but ranged from a low of 7.1% to a high of 62.1% by FISH analysis. These results indicate that there are significant levels of tumor cells within the CP samples and significant levels of normal cells within the AP/BC samples that are not detected by cytogenetic analysis. This is primarily due to the fact that cytogenetic analysis is restricted to cells that are actively dividing in a sample population. Because of the quiescent nature of the purified HSC population, it is impossible to determine the Ph content of these populations by cytogenetics.[37-39] FISH analysis, as outlined in this study, is performed directly on the purified cell populations in the absence of further culture or manipulation. In addition, from the FISH analysis of the purified samples, it is clear that the levels of contaminating tumor cells are significantly different in the two patient populations. Samples from patients in CP

Table 4. Engraftment of MPB Subpopulations in SCID-hu Bone Mice

Experiment No.	Disease Stage	Injected Cell Population*		Cell Number per Graft†	% Positive Grafts (positive grafts/total grafts)‡	%Ph$^+$ Cells in All Grafts (Ph$^+$ cells/total cells)§
		Phenotype	%Ph$^+$ Cells			
1	CP	CD34$^+$	ND	100,000	0.0 (0/10)	55.3 (78/141)
				500,000	75.0 (3/4)	
				1,000,000	50.0 (2/4)	
2	AP	CD34$^+$ Thy-1$^+$ Lin$^-$	78.0	50,000	66.7 (4/6)	60.0 (45/75)
				100,000	100.0 (2/2)	

* Phenotype of the cell population injected into the SCID-hu grafts.
† Number of cells injected into each SCID-hu graft.
‡ Percentage of positive grafts was determined by dividing the number of grafts with >1% donor cells by the total number of grafts analyzed.
§ Percentage of Ph$^+$ cells was determined by dividing the number of Ph$^+$ cells by the total number of cells analyzed.

Table 5. Multilineage Analysis of Donor Cells Isolated From SCID-hu Bone Mice

Experiment No.	Cell Population Analyzed*	Graft Number	% Cells in Graft†	% Cells‡		
				CD34+	CD19+	CD33+
1	Donor	1	40.9	ND	ND	ND
		2	33.8	ND	ND	ND
		3	9.0	ND	ND	ND
		4	28.5	ND	4.7	91.4
		5	6.2	90.0	7.4	94.6
		Mean	23.7	90.0	6.1	93.0
2	Donor	1	19.8	37.1	2.3	84.8
		2	46.2	20.7	0.5	94.7
		3	14.6	44.0	4.8	87.7
		4	10.1	36.5	4.5	80.4
		5	12.4	23.1	6.1	71.5
		6	23.5	14.3	3.7	74.4
		Mean	21.1	29.3	3.7	82.3
	Host	1	80.2	13.4	79.9	7.3
		2	53.8	11.7	76.7	9.6
		3	85.4	12.9	82.9	8.8
		4	89.9	13.5	84.9	7.7
		5	87.6	18.3	73.7	4.2
		6	76.5	10.4	51.1	6.3
		Mean	78.9	13.4	74.9	7.3

* Cell population analyzed is based on HLA class I antibodies that distinguish the donor cells from the host cells.

† Percentage of the cell population being analyzed in the graft (donor + host = 100%).

‡ Percentage of the cell population being analyzed that are CD34+, CD19+, or CD33+.

contained significantly fewer leukemic cells than samples from patients in AP or BC for unpurified (P = .005), CD34+Thy-1+Lin− purified (P = .0005), and CD34+Thy-1−Lin− purified (P = .0029) cell populations. The clinical potential for use of such cell populations as autografts has not yet been tested. However, Talpaz et al[28] recently reported a direct correlation between the numbers of Ph+ cells within an autograft and the levels of Ph+ cells present in the marrow following autologous transplantation of CML patients.

We found that subfractionation of the CD34+Lin− population from the MPB of CML patients resulted in significant levels of residual leukemia cells in the CD34+Thy-1+Lin− population, with mean Ph+ frequencies of 19.2% and 59.2% for the CP and AP/BC samples, respectively. Samples from patients in CP contained significantly fewer leukemia cells in unpurified (P = .005), CD34+Thy-1+Lin− purified (P = .0005), and CD34+Thy-1−Lin− purified (P = .0029) cell populations compared with samples from patients in AP or BC. From these data, one would anticipate that the reduction of Ph+ cells resulting from the isolation of the CD34+Thy-1+Lin− subpopulation could result in a potentially superior autograft.

Results from the in vitro and in vivo characterization of the stem cell activity of these purified cell populations indicate that a population of normal HSC are present in the MPB of CP CML patients. Long-term CAFC analysis of subpopulations of CD34+ cells indicates the presence of pluripotent progenitor cells within the CD34+Thy-1+Lin− population of MPB collected from both CP and AP/BC CML patients. The CAFC frequency of the CD34+Thy-1+Lin− population was similar to that detected in the MPB collected from patients with a variety of malignancies.[24] Although the frequency of CAFC is lower in the CD34+Thy-1−Lin− population, a significant number of CAFC are present in this cell population, the majority of which are likely due to leukemic stem cells. In vivo engraftment of the purified cell populations in the SCID-hu bone assay lends further support to the existence of both normal and leukemic HSC within these populations. In both experiments, when sufficient cells were injected in each graft, a majority of the grafts showed multilineage donor engraftment after 8 weeks.

These studies indicate that the SCID-hu bone assay can be used to assess the engraftment potential of purified HSC populations. Elimination or reduction of the leukemic burden will rely on further attempts to subfractionate the CD34+ population using additional stem cell markers or elimination of the tumor using markers found on a majority of leukemic cells. Reports of autologous transplantation of CML patients using unfractionated MPB cells indicate that many patients are in cytogenetic remission for a limited period of time following autotransplantation.[6,27,28,40] Because these grafts must certainly contain leukemic cells, it is hopeful that further reductions in the levels of reinfused tumor cells may result in prolonged periods of remission.

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