Selection of Benign Primitive Hematopoietic Progenitors in Chronic Myelogenous Leukemia on the Basis of HLA-DR Antigen Expression

By Catherine M. Verfaillie, Wesley J. Miller, Kristin Boylan, and Philip B. McGlave

Chronic myelogenous leukemia (CML) is a lethal malignancy of the human hematopoietic stem cell. Here we report that coexistent benign, primitive hematopoietic progenitors can be distinguished from their malignant counterparts in CML bone marrow by differences in cell surface antigen expression. Selection of bone marrow cells expressing the CD34 antigen but lacking the HLA-DR antigen results in recovery of secondary clonogenic cells derived at week 1, 5, and 8 from long-term bone marrow cultures (LTBMCs) initiated with primitive progenitors, which lack HLA-DR antigens, exhibit neither the Philadelphia chromosome (Ph') nor the corresponding bcr/abl mRNA characteristic of CML. In contrast, clonogenic cells recovered at week 1, 5, and 8 from LTBMCs initiated with the CML HLA-DR⁺ population contain Ph' and express bcr/abl mRNA. This observation indicates that it may be possible to select a population of viable, exclusively benign hematopoietic stem cells from CML bone marrow capable of repopulating the hematopoietic compartment following autologous bone marrow transplantation.

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MATERIALS AND METHODS

Bone Marrow Samples

Bone marrow was obtained from the posterior iliac crest of seven chronic-phase CML patients and 10 healthy young volunteers after informed consent. Patient characteristics are summarized in Table 1.

Bone marrow mononuclear cells (BMMNC) were obtained after Ficoll-hypaque separation (specific gravity, 1.077). BMMNC from patients 5 to 7 were purified further in an initial counterflow elutriation step. BMMNC were resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal calf serum (FCS) and 0.01% EDTA. The cells were injected into an elutriator system with standard separation chamber (Beckman Instruments, Palo Alto, CA) primed with IMDM-FCS-EDTA. Rotor speed and temperature were maintained at 1,950 rpm and 10°C. After loading, 200 mL of effluent was collected at a flow rate of 8 mL/min, and 100 mL was collected at a flow rate of 12 mL/min. The rotor was then stopped and the remaining BMMNC flushed from the separation chamber.

Purification of Progenitor Populations

Purified progenitor populations were obtained using previously described methods. In short, BMMNC (patients 1 to 4, normals) were first elutriated. The resulting population contained cells capable of forming hematopoietic colonies in vitro, including progenitors expressing the CD34 antigen. These cells were then subjected to a second elutriation step, which resulted in the recovery of cells with the phenotype CD34⁺/HLA-DR⁻. The remaining CD34⁻/HLA-DR⁺ lineage-negative cells were then used as a control population.

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counterflow elutriation were labeled with 25 ng/10⁶ cells anti-CD2, with low levels of lineage-specific mouse antibodies. The resultant lineage-negative cells were labeled with anti-CD34 antibody (250 ng/10⁶ cells) (Becton Dickinson), followed by fluorescein isothiocyanate (FITC)-conjugated goat F(ab)² anti-mouse IgG, and finally mouse IgG₂,₂-PE antibodies (Fig 1B). Less than 0.1% residual cells labeled with low levels of lineage-specific mouse IgG used in the lineage-negative immunomagnetic depletion step were included in the CD34⁻ fraction. Analysis of sorted cells demonstrated that 97% or more of DR⁻ cells expressed CD34 antigens and 2% or less expressed HLA-DR antigens, while DR⁺ cells contained 95% or more cells expressing both CD34 and HLA-DR antigens. Although both DR⁻ and DR⁺ cells were sorted using the same lightscatter gates, the lightscatter profiles from reanalyzed sorted cells indicated that DR⁻ cells are slightly smaller (very low forward scatter) than those sorted in the DR⁺ fraction (low forward scatter).

**Short-Term Methylcellulose Cultures**

DR⁻ and DR⁺ cells (10⁶ cells/mL) were plated in clonogenic methylcellulose assay as previously described, containing 3 IU recombinant erythropoietin (Amgen, Thousand Oaks, CA) and 10% conditioned media from the bladder carcinoma cell line 5637. Cultures were incubated in a humidified atmosphere at 37°C and 5% CO₂, and assessed for the presence of colony-forming cells (CFC) (mixed colony-forming unit [CFU-MIX], granulocyte-macrophage CFU [CFU-GM], and burst-forming unit–erythroid [BFU-E]) at day 21. The absolute number of CFC recovered in a certain fraction was calculated as the number of CFC/10⁶ cells of that fraction multiplied by the total number of cells recovered in that fraction.

**LTBMC**

DR⁻ (10⁶ cells/mL) and DR⁺ (10⁶ cells/mL) cells were plated on allogeneic irradiated stroma in LTBMC media as previously described. Cultures were maintained in a humidified atmosphere at 37°C and 5% CO₂. At weekly intervals, the cultures were fed by removing half of the supernatant and replacing it with fresh media. Nonadherent cells recovered in the supernatant, as well as adherent cells recovered from selected stromal layers after treatment.
PRIMITIVE PROGENITORS IN CML ARE HLA-DR*

with 0.1% collagenase, were plated in short-term methylcellulose culture and colonies enumerated at day 14 to 16 of culture. It is believed that committed cells present in the initial bone marrow population fail to produce secondary clonogenic cells in LTBM C 4 to 5 weeks after initiation of LTBM C.10-12 The number of secondary clonogenic cells recovered from both adherent and nonadherent layers of LTBM C at 5 weeks of cultures therefore represents progeny of more primitive progenitors, which can initiate long-term in vitro hematopoiesis and are termed LTBM C initiator cells or LTBM C-IC.9,10 The absolute number of LTBM C-IC recovered in a certain fraction was calculated as the number of LTBM C-IC/10^5 cells of that fraction multiplied by the total number of cells recovered in that fraction.

Cytogenetic Analysis of Plucked Colonies

Colonies plucked from short-term methylcellulose cultures of cells harvested from adherent and nonadherent fractions of LTBM C initiated with CML DR* and DR+ progenitors, respectively, were pooled in IMDM with 20% FCS and were subjected to a 1.5-hour colcemid incubation followed by lysis with hypotonic KCl and fixation in acid/alcohol as previously described. In each of seven cases described, metaphases were analyzed after QFQ or GTG banding.16

Polymerase Chain Reaction (PCR) Amplification of the bcr/abl mRNA

Colonies arising from adherent and nonadherent cells of LTBM C initiated with CML DR* and DR+ cells, respectively, were plucked from methylcellulose cultures using a Pasteur pipette and frozen at −70°C in 100 μL phosphate-buffered saline (PBS).

RNA preparation. Total cellular RNA was extracted from thawed colonies according to the method of Chomczynski and Sacchi.17 To protect the RNA, guanidinium was added before cell disruption. Pelleted cells were lysed in the presence of guanidinium thiocyanate, sodium citrate, and sarcosyl, and RNA was extracted with phenol and chloroform/isoamyl alcohol. RNA was precipitated using glycogen as a carrier.

Oligonucleotides. Oligonucleotide primers and probes were prepared using a Biosearch 6800 DNA synthesizer18-21: (a) 5'-GGAGC-GCT-GCA-ACT-GTC-CAG-ACC-ATG-CCG-3' (outer bcr 5' sense primer); (b) 5'-CTC-AGC-GCT-CAG-ACC-ATG-CCG-3' (outer bcr 3' antisense primer); (c) 5'-GGAGC-GCT-GCA-ACT-GTC-CAG-ACC-ATG-CCG-3' (inner bcr 5' sense primer); (d) 5'-CTC-AGC-GCT-CAG-ACC-ATG-CCG-3' (inner bcr 3' antisense primer); (e) 5'-GCT-GAA-GGG-C'TT-CCT-TAT-TGA-TG-3' (bcr exon 2/abl exon 2 splice) (B3/A2) or 166 bp DNA ladder. RNAs were purified using a Qiagen RNeasy kit, and total cellular RNA was extracted from the following cell lines: K562 (control for B2/A2 splice; cells from a patient with known breakpoint 5' to bcr exon 3: control for B3/A2 splice) and negative (Raji cells and a reagent control consisting of all reagents except RNA) controls. To interpret negative results, RNA samples were subjected to PCR using probes for β-actin mRNA22 using the following oligonucleotides: (g) 5'-TAC-CTG-AGG-AAG-TGC-CTC-A-3'; (h) 5'-AGG-AGC-CAG-CTA-TGG-GAA-TTG-TAT-TGT-AG-3' (i) 5'-CCA-TTC-CTT-GCT-GAC-AGT-3'.

Sequences (g) and (h) are the 5' sense primer from exon III, (h) is the 3' antisense primer from exon IV, and (i) is the internal probe representing an exon III antisense sequence. Amplification of β-actin mRNA was accomplished using conditions identical to the bcr/abl amplification, except that oligonucleotide (h) was used as primer for reverse transcription, oligonucleotide (g) added with Taq polymerase, and following electrophoresis and Southern transfer, the β-actin cDNA probe with oligonucleotide (i). The resulting amplified cDNA was 273 bp.

Statistics

Results of experimental points obtained from multiple experiments are reported as the mean ± 1 SEM. Significance levels were determined by two-sided Student's t-test.

RESULTS

Purified progenitor populations were obtained by depleting BMMNC of cells expressing myeloid, monocytic, B-lymphoid, T-lymphoid, and natural killer cell antigens by immunomagnetic depletion (lineage-negative). The lineage-negative cells were subsequently subjected to positive selection by four parameter FACS sorting of cells with low/very low horizontal light scatter properties and very low vertical light scatter properties, expressing high density of CD34 antigens and either expressing (referred to as DR+ cells) or lacking (referred to as DR cells) HLA-DR antigen (Fig 1).10 The DR+ and DR− cell content of CML BMMNC was significantly lower than that of 10 normal individuals (Table 2). This was accompanied by an increase in more mature differentiated myeloid cells, which were depleted during the initial negative immunomagnetic selection step. The morphology of CML DR cells is that of favor denaturation (95°C for 20 seconds), annealing (55°C for 15 seconds), and primer extension (72°C for 1 minute). A 10-μL aliquot of this PCR solution was subsequently added to 87 μL of PCR buffer, dNTP, 5' and 3' primers (b [bcr 5' sense primer] and c [abl 3' antisense primer]) internal to the first set of primers and Taq polymerase. This second solution underwent 30 cycles on the DNA Thermal Cycler.22

Detection of amplified fragments. Amplified samples were size-separated by gel electrophoresis using 3% NuSieve, 1% Seakem (FMC BioProducts, Rockland, ME) gel. A 123-bp DNA ladder (Bethesda Research Laboratories) was used as a size marker. Gels were stained with ethidium bromide and photographed. Amplified cDNA fragments were transferred electrophoretically to a Nitran nylon membrane (Schleicher & Schuell, Keene, NH). Antisense oligonucleotides complementary to the B2/A2 splice and B3/A2 splice were end-labeled using tdt (15 U/μL) (Bethesda Research Laboratories), 3P-dATP-Cordycepin (DuPont/New England Nuclear, Wilmington, DE), and 1 to 2 x 106 cpm/mL added to the hybridizing solution for each membrane. Following 12 to 16 hours of hybridization at 42°C, membranes were washed. After initial hybridization with the B2/A2 probe, all membranes were stripped and reprobed with the B3/A2 probe.

Controls. To test for contamination, we included several positive (K562: control for B2/A2 splice; cells from a patient with known breakpoint 5' to bcr exon 3: control for B3/A2 splice) and negative (Raji cells) and a reagent control consisting of all reagents except RNA) controls. To interpret negative results, RNA samples were subjected to PCR using probes for β-actin mRNA22 using the following oligonucleotides: (g) 5'-TAC-CTG-AGG-AAG-TGC-CTC-A-3'; (h) 5'-AGG-AGC-CAG-CTA-TGG-GAA-TTG-TAT-TGT-AG-3'; (i) 5'-CCA-TTC-CTT-GCT-GAC-AGT-3'.

Sequence (g) is the 5' sense primer from exon III, (h) is the 3' antisense primer from exon IV, and (i) is the internal probe representing an exon III antisense sequence. Amplification of β-actin mRNA was accomplished using conditions identical to the bcr/abl amplification, except that oligonucleotide (h) was used as primer for reverse transcription, oligonucleotide (g) added with Taq polymerase, and following electrophoresis and Southern transfer, the β-actin cDNA probe with oligonucleotide (i). The resulting amplified cDNA was 273 bp.

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small lymphocyte-like blasts contaminated with small numbers of basophilic precursors, while CML DR+ cells are larger blasts with multiple nucleoli.

To determine the number of committed clonogenic cells in CML DR- and DR+ cells, we plated both cell fractions in short-term methylcellulose assay. The number of clonogenic cells present in the CML DR- population was similar to that found in the DR+ fraction from normal individuals (3.5 \pm 1.3/10^6 CML BMMNC) was significantly lower than the absolute number of DR- clonogenic cells in normal bone marrow (58.3 \pm 13.2/10^6 normal BMMNC, P = .004). The number of clonogenic cells present in the DR+ population derived from CML bone marrow was similar to that found in normal DR+ cells. As was seen for the DR- clonogenic cells present in CML bone marrow, the absolute number of DR- clonogenic cells in CML bone marrow (198.9 \pm 88/10^6 BMMNC) was significantly different than that in normal bone marrow (542 \pm 52/10^6 BMMNC, P = .003). This lower absolute number of both DR- and DR+ clonogenic cells recovered from CML bone marrow is possibly the result of the increase in more mature committed myeloid progenitors characteristic of CML.

We then evaluated the number of more primitive LTBMC-IC present in either cell population by plating DR- and DR+ cell fractions on irradiated allogeneic normal bone marrow stroma. These cultures were then evaluated for the production of secondary clonogenic cells in the nonadherent supernatant and the adherent stromal layers over 8 consecutive weeks (Table 2, Fig 2A through D). Similar to normal DR- cells (Fig 2B), CML DR- cells (Fig 2A) are capable of inducing long-term hematopoiesis in LTBMC for at least 8 weeks. Likewise, the absolute number of DR+ LTBMC-IC present in CML bone marrow (25.9 \pm 9.1/10^6 CML BMMNC) was significantly greater than that of normal bone marrow (5.03 \pm 1.1/10^6 normal BMMNC, P = .02).

To determine the clonal origin of primitive progenitors in CML DR- and DR+ cells, we plated DR- and DR+ cells from CML bone marrow on irradiated allogeneic stroma derived from normal donors of the opposite sex. These LTBMC cultures were killed at week 1, 5, or 8 and recovered cells plated in methylcellulose culture. Resultant secondary colonies were plucked and examined for the presence of the Ph+ and expression of bcr/abl mRNA. Cytogenetic studies\(^*\) of pooled, plucked secondary colonies obtained from LTBMC initiated with DR- cells of six CML patients (patients 1 to 6) demonstrated only normal karyotype at week 1, 5, and 8 of culture (Table 3). The benign or malignant nature of these clonogenic cells was confirmed by molecular genetic analysis of PCR-amplified total cellular mRNA of single colonies plucked at week 5 for patients 3, 5, and 6 and at week 8 for patient 4 (Table 3, Fig 3). All secondary colonies derived from LTBMC initiated with DR+ cells from patients 3, 4, 5, and 8 failed to express the bcr/abl mRNA, but contained amplifiable \(\beta\)-actin mRNA. In contrast to the previous six patients, all but one secondary colonies derived from LTBMC initiated

\*DR- or DR+ cells were obtained after sequential negative immunomagnetic depletion of cells of committed myeloid/lymphoid lineage (lineage-negative cells represented 1.9% \pm 1.4% of CML BMMNC and 5.8% \pm 0.48% of normal BMMNC and FACS sort for CD34+/HLA-DR- and CD34+/HLA-DR+ cells.

\#Comparison of DR- and DR+ cells in CML, P < .05.

<table>
<thead>
<tr>
<th>Bone Marrow Population</th>
<th>% of BMMNC</th>
<th>Clonogenic Progenitors/10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML patients (n = 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td>0.02 \pm 0.01††</td>
<td>1,411 \pm 5929</td>
</tr>
<tr>
<td>DR+</td>
<td>0.18 \pm 0.06†</td>
<td>9,861 \pm 1,426</td>
</tr>
<tr>
<td>Normals (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td>0.24 \pm 0.03</td>
<td>2,240 \pm 280</td>
</tr>
<tr>
<td>DR+</td>
<td>0.59 \pm 0.05</td>
<td>7,670 \pm 660</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. Characteristics of CML and Normal DR- and DR+ Cells</th>
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Table 3. Clonal Origin of Secondary Colonies Derived From LTBMC Initiated With DR- or DR' Cells Obtained From CML Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>LTBMC (wk)</th>
<th>DR- Cells</th>
<th>Metaphases</th>
<th>Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ph'-Positive/Total</td>
<td>bcr/abl-Positive/Total</td>
</tr>
<tr>
<td>1*</td>
<td>1</td>
<td>0/3</td>
<td>NT</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/6</td>
<td>NT</td>
<td>NM</td>
</tr>
<tr>
<td>2*</td>
<td>1</td>
<td>0/20</td>
<td>NT</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/20</td>
<td>0/4</td>
<td>16/16</td>
</tr>
<tr>
<td>3*</td>
<td>1</td>
<td>NM</td>
<td>NT</td>
<td>NM</td>
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<td></td>
<td>5</td>
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<td>0/4</td>
<td>1/1</td>
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<td>4*</td>
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<td>NM</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0/11</td>
<td>0/4</td>
<td>1/1</td>
</tr>
<tr>
<td>5*</td>
<td>1</td>
<td>0/13</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/20</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0/9</td>
<td>NT</td>
<td>5/5</td>
</tr>
</tbody>
</table>

Abbreviations: NM, no metaphases detected; NT, not tested.
*Male patient on female stroma; all analyzed karyotypes demonstrated male sex.
†Female patient on male stroma; all analyzed karyotypes demonstrated female sex.

with DR- cells from patient 7 contained the Ph', and eight of nine analyzed colonies recovered at week 5 expressed the \( bcr/abl \) mRNA.

Secondary colonies obtained from LTBMC initiated with DR' cells from patients 1, 2, 3, 4, 5, and 7 demonstrated only Ph'-positive metaphases (Table 3), and this at week 1, 5, and 8 of culture. All analyzed secondary colonies from 5- or 8-week-old LTBMC initiated with DR' cells of patients 3, 4, 5, and 7 also expressed the \( bcr/abl \) mRNA (Table 3, Fig 3). The \( bcr/abl \) splicing pattern demonstrated in the amplified mRNA from these colonies was consistent with the \( bcr \) breakpoint location and the splicing pattern seen in bone marrow from the same patient. In contrast to the previous patients, secondary colonies recovered at week 5 from LTBMC initiated with DR' cells of patient 6 demonstrated only normal karyotype and only four of 10 analyzed colonies contained \( bcr/abl \) mRNA, while all but one analyzed colonies recovered at week 1 of LTBMC initiated with DR' cells contained the Ph'.

**DISCUSSION**

This study demonstrates that coexistent benign, primitive hematopoietic progenitors can be separated from their malignant counterparts in CML bone marrow by differences in cell surface antigen expression. These benign primitive progenitors are phenotypically similar to very primitive hematopoietic progenitors isolated from the bone marrow of normal individuals. Such cells are morphologically small blasts, exhibit CD34 antigen expression, and lack detectable levels of antigens associated with T-cell, B-cell, or myeloid lineage, as well as HLA-DR antigens. These benign DR' primitive progenitors from CML bone marrow proliferate in stroma-dependent cultures for at least 8 weeks. This population can be distinguished from malignant Ph'-positive primitive progenitors with long-term in vitro hematopoietic repopulating capacity by differences in cell surface antigen expression (HLA-DR expression).

The number of secondary clonogenic progenitors cultured in LTBMC initiated with CML DR- cells is significantly lower than that of LTBMC initiated with normal DR- cells. This is unlikely to be the result of more extensive
contamination of the CML DR- cell fraction with more mature committed progenitors, as the number of committed clonogenic cells in CML DR- cells is not different than that in normal DR+ cells. This observation may indicate that primitive progenitor cells present in the CML DR- cell population are more quiescent or respond less well to growth signals generated in normal irradiated stromal layers compared with a similar population of DR+ cells derived from normal bone marrow.

The absolute number of LTBMC-IC, measured as secondary clonogenic cells in LTBMC initiated with DR- cells multiplied by the percent recovery of cells in the DR- fraction, present in CML bone marrow mononuclear cells is significantly lower than that in normal bone marrow. This may be the result of a more quiescent state of benign primitive progenitors in CML bone marrow compared with primitive progenitors found in normal bone marrow. Alternatively, these observations may suggest that the number of benign primitive progenitors in CML bone marrow is significantly lower than that of normal bone marrow. Additional studies in which the absolute number of LTBMC-IC, rather than LTBMC-IC-derived secondary clonogenic cells, is evaluated will be needed to resolve this question.

Previous studies have demonstrated that growth of progenitors from CML bone marrow ex vivo in long-term marrow culture results in the selection of coexistent benign Ph'-negative progenitors. In these studies, clonogenic and granulocytic cells recovered from LTBMC initiated with CML BMNC exhibit the Ph+ and bcr/abl gene rearrangement through week 4 to 5 of culture, whereas cells recovered after week 4 to 5 contained only Ph+-negative nonclonal progenitors. In the present study, we demonstrate that CML DR+ cells give rise exclusively to benign Ph'-negative clonogenic progeny in both nonadherent and adherent fractions of LTBMC culture as early as day 7 after initiation of the culture. Moreover, culture of the DR+ fraction of bone marrow resulted in the persistent generation of malignant Ph-positive secondary clonogenic cells through week 8 of culture. These data indicate that the malignant Ph-positive clone derived from the bone marrow of these CML patients can persist in LTBMC when cultured separately. The presence of exclusively benign clonogenic cells as early as 7 days after initiation of the ex vivo culture in LTBMC initiated with CML DR+ cells is therefore unlikely to be the result of an in vitro selection attributable to the LTBMC culture itself, but of the selection by phenotype performed before culture in LTBMC. Analysis of pooled colonies for the presence of the Ph+ chromosome may skew the results in the direction of more highly mitotically active colonies in the pool, and may decrease the sensitivity to detect nondividing, possibly malignant, clonogenic cells. Similarly, evaluation of a relatively small number of single plucked second generation colonies (four to 11 colonies per patient) for the presence of the bcr/abl gene rearrangement may not detect a minor population of malignant colonies. However, the consistently benign genotype of the DR+ population in six of seven patients indicates that selection of the DR+ fraction from CML bone marrow results in the purification of a population of exclusively benign primitive progenitors.

The presence of benign secondary clonogenic cells in LTBMC initiated with DR+ cells from patient 6 at week 5 of LTBMC culture may be the result of the long-term culture itself, rather than the initial selection process. Patient 6 differed from the other patients in that he was recovering from severe bone marrow aplasia induced by hydroxyurea therapy. Cytogenetic studies of freshly aspirated bone marrow cells obtained at the time of study showed a predominance (67%) of Ph'-negative cells. The DR+ cells from this patient failed to sustain generation of secondary clonogenic progenitors in LTBMC for more than 5 weeks. The benign Ph'-negative progenitors present in the DR+ cell population may therefore represent an unusually expanded population of less primitive benign progenitors with limited self-renewing capacity.

The only patient in whom the DR+ fraction of bone marrow cells contained malignant Ph-positive progenitors is patient 7. Clinically, patient 7 differs from the other six patients in that she was treated with interferon-α until 3 months before study and with hydroxyurea until the day of study. Recent studies have demonstrated that interferon-α can restore the impaired adhesion of CML progenitors to the bone marrow microenvironment and may render these progenitors susceptible to negative regulatory signals produced by marrow stromal cells, which maintain the stem cell reserve in a quiescent state. Moreover, hydroxyurea inhibits ribonucleotide reductase, resulting in depletion of the deoxyribonucleotide pools required for DNA synthesis and inhibition of cell replication. This prevents malignant CML progenitors from cycling and may decrease their expression of cell surface activation antigens, such as HLA-DR.

Multiple studies have addressed the expression of HLA-A, B, and D antigens in human malignancies. De novo or increased expression of class II HLA antigens on malignant cells has been observed in a variety of tumors. It has been speculated that downregulation or upregulation of HLA-class I or II expression may be a common feature of malignant transformation mediated by oncogenes. Class I HLA antigen expression can be influenced by oncogenic viral infection or oncogene transfection into normal cells with resultant cell transformation. HLA-DR expression is increased after malignant transformation of melanocytes with ras oncogenes. The increased expression of HLA-DR antigens on malignant CML primitive progenitors could therefore be caused by dysregulation of the HLA-DR gene or its expression-regulating genes by the bcr/abl gene product. Similar to what we observe here for CML DR+ cells, we demonstrated previously that the less primitive DR+ cells derived from normal human bone marrow fail to adhere to irradiated stromal layers. HLA-DR expression on the malignant DR+ cell fraction could therefore be a reflection of a more differentiated stage of these progenitors compared with the benign DR+ fraction in normal or CML bone marrow. This would be consistent with the observation that more differentiated cells within the same lineage often express greater density of HLA-DR antigens. However, the observation that such malignant DR+ progenitors give rise to sustained in vitro generation of clonogenic progenitors indicates that benign DR- and
malignant DR' primitive progenitors have similar self-renewing capacity and may therefore represent two coexisting cell populations at the same stage of differentiation. It has been demonstrated that malignant Ph'-positive primitive clonogenic progenitors in CML bone marrow show an increased rate of turnover compared with their quiescent DR' counterparts in normal individuals. Since HLA-DR antigen expression is cell cycle-related and increases when cells evolve from G0 to G1,

the increased expression of HLA-DR antigens may be caused by differences in cell activation status. This could either be induced by the increased tyrosine kinase activity of the bcr/abl gene product, which may allow CML progenitors to grow in reduced quantities, or in the absence of specific growth factors. Alternatively, since primitive malignant CML progenitors adhere significantly less to elements from the bone marrow microenvironment, they may escape from the inhibitory effects of negative hematopoietic regulators such as transforming growth factor-β (TGF-β), which are generated locally in the bone marrow microenvironment and retain primitive hematopoietic progenitors in a noncycling, resting state.

Further evaluation of differences between the benign and malignant primitive hematopoietic progenitor population derived from CML bone marrow may lead to a better understanding of the uncontrolled proliferation of the malignant stem cell clone observed in CML and of the abnormal distribution of malignant myeloid precursors in the peripheral blood of patients with CML. The ability to purify both benign and malignant primitive progenitors from CML bone marrow will provide us with a powerful tool to perform such studies. Currently, allogeneic bone marrow transplantation is the only available curative therapy for CML. This study suggests that it may be feasible to select a population of viable, benign hematopoietic stem cells from the bone marrow of certain CML patients, which could be used for autologous bone marrow transplantation in patients without suitable allogeneic bone marrow donors.

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REFERENCES

chronic myelocytic cell line K562 contains a breakpoint in bcr and produces a chimeric bcr/c-abl transcript. Mol Cell Biol 6:607, 1986
40. Verfaillie CM, McCarthy JB, McGlave PB: Mechanisms underlying abnormal trafficking of malignant progenitors in chronic myelogenous leukemia: Decreased adhesion to stroma and fibronectin but increased adhesion to the basement membrane components laminin and collagen type IV. Blood 78:172a, 1991 (abstr, suppl 1)
Selection of benign primitive hematopoietic progenitors in chronic myelogenous leukemia on the basis of HLA-DR antigen expression

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