High Level Engraftment of NOD/SCID Mice by Primitive Normal and Leukemic Hematopoietic Cells From Patients With Chronic Myeloid Leukemia in Chronic Phase


We have previously shown that intravenously injected peripheral blood (PB) or bone marrow (BM) cells from newly diagnosed chronic myeloid leukemia (CML) patients can engraft the BM of sublethally irradiated severe combined immunodeficient (SCID) mice. We now report engraftment results for chronic phase CML cells in nonobese diabetic (NOD)/SCID recipients which show the superiority of this latter model. Transplantation of NOD/SCID mice with 7 to 10×10^7 patient PB or BM cells resulted in the continuing presence of human cells in the BM of the mice for up to 7 months, and primitive human CD34^+ cells, including those detectable as colony-forming cells (CFC), as long-term culture-initiating cells, or by their coexpression of Thy-1, were found in a higher proportion of the NOD/SCID recipients analyzed, and at higher levels than were seen previously in SCID recipients. The human CFC and total human cells present in the BM of the NOD/SCID mice transplanted with CML cells also contained higher proportions of leukemic cells than were obtained in the SCID model, and NOD/SCID mice could be repopulated with transplants of enriched CD34^+ cells from patients with CML. These results suggest that the NOD/SCID mouse may allow greater engraftment and amplification of both normal and leukemic (Ph^+) cells sufficient for the quantitation and characterization of the normal and leukemic stem cells present in patients with CML. In addition, this model should make practical the investigation of mechanisms underlying progression of the disease and the development of more effective in vivo therapies.

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or umbilical cord blood cells. However, we also found that the level of human hematopoiesis obtained in SCID mice was generally low, regardless of the source of the injected cells, and even with very large transplants of CML cells, only a minority of the cells later found to be present in the mice were leukemic. Subsequently we discovered that nonobese diabetic (NOD)/SCID mice, which have additional defects in natural killer (NK) cell activity as well as defective macrophage and complement function, allow superior engraftment of normal and leukemic human hematopoietic cells. It therefore seemed likely that these mice might also prove to be better recipients of CML cells. We now describe an improved in vivo model for CML, using the NOD/SCID mouse as a recipient. By comparison with the SCID model, engraftment of NOD/SCID mice with multiple types of normal and leukemic human cells was higher and could be achieved with lower numbers of CD34+ cell–enriched populations. These experiments provide a foundation for the future characterization of the phenotype and properties of normal and Ph+ cells that have long-term in vivo repopulating activity, as well as for the development of strategies to selectively manipulate normal and Ph+ stem cell populations in vivo.

MATERIALS AND METHODS

Patient cells. BM and PB samples were obtained from patients with informed consent according to procedures approved by the Human Experimentation Committee at the Princess Margaret Hospital, Toronto: the Toronto Hospital; and the Vancouver Health Sciences Hospital. All patients had Ph+ CML and were in chronic phase at the time the sample was taken (Table 1). Fresh PB or BM was diluted 1:2 with Iscove’s modified Dulbecco’s medium (IMDM; GIBCO-BRL, Burlington, Ontario, Canada) containing 10% fetal calf serum (FCS) and enriched for mononuclear cells by Ficoll density gradient centrifugation. CD34+ cells were then selected from some of these samples by using QBEnd10, an antibody that detects a class II CD34 epitope (generously provided by Dr Hornby, Ontario, Canada) under conditions that efficiently removed patients with Ph+ CML and were in chronic phase at the time the sample was taken. 

Table 1. Clinical Data for Patients’ Samples Used in This Study

<table>
<thead>
<tr>
<th>Patient No.*</th>
<th>Age</th>
<th>Sex</th>
<th>PB WBC Count (+10%)</th>
<th>Prior Treatment</th>
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<td>2</td>
<td>26</td>
<td>M</td>
<td>395</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>M</td>
<td>45</td>
<td>Hydroxyurea × 2 mos</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>M</td>
<td>58</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>F</td>
<td>160</td>
<td>None</td>
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<tr>
<td>6</td>
<td>48</td>
<td>M</td>
<td>135</td>
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<td>M</td>
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<td>51</td>
<td>F</td>
<td>135</td>
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<td>None</td>
</tr>
<tr>
<td>13</td>
<td>72</td>
<td>M</td>
<td>218</td>
<td>None</td>
</tr>
<tr>
<td>14</td>
<td>63</td>
<td>M</td>
<td>123</td>
<td>None</td>
</tr>
<tr>
<td>15</td>
<td>37</td>
<td>M</td>
<td>82</td>
<td>Hydroxyurea × 1 wk</td>
</tr>
<tr>
<td>16</td>
<td>34</td>
<td>F</td>
<td>120</td>
<td>None</td>
</tr>
<tr>
<td>17</td>
<td>63</td>
<td>M</td>
<td>87</td>
<td>One 4-wk course of busulphan 30 mos previously</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female.

*All patients had Ph+ CML and were in chronic phase at the time the sample was taken.

RESULTS

Engraftment of NOD/SCID mice by BM and PB cells from patients with chronic phase CML. Fresh or previously frozen light density cells from the BM or PB of 11 patients with chronic phase CML (Table 1) were transplanted by tail vein injection into sublethally irradiated NOD/SCID mice. The extent of human cell engraftment in the BM of these mice was determined by Southern blotting using a human-specific DNA probe, or by flow cytometric detection of cells expressing the phycoerythrin from 10^6 KGlA cells suspended in 50 µl of RPMI within 20 minutes at 37°C. The percentage of CD34+ cells in the glycoprotease–selected fraction was determined by flow cytometry using antibodies to glycoprotease-resistant epitopes of CD34 and CD45, and light scatter, as described. In some experiments, unseparated or CD34-enriched cells were suspended in 10% dimethyl sulfoxide, frozen at −70°C, stored in liquid nitrogen until required, and then thawed before transplantation into mice.

Analysis of mice. NOD/SCID mice were bred and maintained in the defined flora animal colony at the Ontario Cancer Institute, Toronto. Unless otherwise stated, 7.5 to 10 × 10^6 light density cells or various numbers of enriched CD34+ cells were injected into the tail vein of sublethally irradiated (400 cGy using a 137Cs γ-irradiator) 8-week-old NOD/SCID mice. Some mice also received various combinations of intraperitoneally injected human cytokines on alternate days as indicated. Cytokines used included mast cell growth factor (MGF, 10 µg), PIXY321 (7 µg), interleukin-3 (IL-3, 6 µg), granulocyte-macrophage colony-stimulating factor (GM-CSF, 6 µg), G-CSF (6 µg), and Flt-3 ligand (FL, 6 to 10 µg) (MGF, PIXY, and FL from Immunex; IL-3, GM-CSF, and G-CSF from Amgen; for the last two experiments, stem cell factor from Amgen was used instead of MGF). Mice were killed 1 to 7 months after transplantation, or as early as 2 weeks posttransplant if they appeared to be sick. The cells present in eight bones (ie, both femurs, tibiae, iliac crests, and humeri) were obtained in IMDM containing 10% FCS. The proportion of all human cells in the BM of transplanted mice was quantified by Southern blot analysis using a human-specific probe as previously described, or by flow cytometry on a FACScan analyzer (Becton Dickinson, San Jose, CA) after staining with human-specific monoclonal antibodies directed against CD45 and CD7. Some cells obtained from the four leg bones of some mice were shipped on wet ice by overnight courier to Vancouver for additional phenotyping studies, isolation of CD34+ cells, plating in CFC and LTC-IC assays, and cytogenetic analyses as reported. In some instances CFC assays on unseparated cells were also performed by using selective conditions that do not allow coexisting mouse CFC to be detected.

BCR Southern analyses. DNA was extracted from cells obtained from the BM of transplanted mice, digested, and separated by gel electrophoresis. Southern blot analysis was then performed using a BglII/HindIII probe that contains the human BCR exon. The relative intensities of the germline and rearranged bands were determined by scanning the developed film on a Computing Densitometer (Model 300A; Molecular Dynamics, Sunnyvale, CA) followed by analysis using ImageQuant software (Version 3.3; Molecular Dynamics). The percentage of leukemic cells was calculated assuming that normal cells contribute two copies to the germline band and leukemic cells contribute one copy each to the germline and rearranged bands. The limit of detection for this assay is approximately 0.1 µg of DNA.

Statistical analysis. Comparisons of the level of engraftment in NOD/SCID versus SCID mice were performed using the Mann-Whitney Rank Sum Test (SigmaStat version 1.0; Jandel Software; Labtromics, Inc, Guelph, Ontario, Canada). Results are expressed as mean ± SEM.

RESULTS

Engraftment of NOD/SCID mice by BM and PB cells from patients with chronic phase CML. Fresh or previously frozen light density cells from the BM or PB of 11 patients with chronic phase CML (Table 1) were transplanted by tail vein injection into sublethally irradiated NOD/SCID mice. The extent of human cell engraftment in the BM of these mice was determined by Southern blotting using a human-specific DNA probe, or by flow cytometric detection of cells expressing the...
human-specific markers CD45 and CD71. DNA results from one experiment are shown in Fig 1A. In several of the mice in this experiment, more than 50% of all the cells in the BM were human, although there was considerable variability in the level of engraftment, even among mice treated identically. The three lanes marked by an asterisk indicate mice that were killed more than 5 months after transplantation, showing the durability of the engraftment. Fig 1B shows the proportion of human cells detected in the BM of 65 mice transplanted with chronic phase CML cells, as determined by flow cytometry using antibodies to CD45 and CD71. The number of cells transplanted per mouse ranged from $7.5 \times 10^7$ to $10^8$ except for two mice that received $2 \times 10^7$ PB cells and two mice that received $8 \times 10^6$ PB cells. (—) Indicate geometric means.
Presence of primitive human hematopoietic cells in the BM of engrafted mice. To determine whether primitive human hematopoietic cells could be detected in the transplanted mice, BM cells from 33 engrafted mice (1% to 80% human cells) were examined by flow cytometry for expression of human Thy-1 and/or CD34. All but one of the mice were analyzed between 2 and 7 weeks; one mouse was sacrificed after 6.5 months. The example shown in Fig 2 is from a mouse that was sacrificed 4 weeks posttransplant; 49% of the viable (PI2) cells in the BM of this mouse were human, as indicated by CD45/CD71 staining (panel A). Approximately 2.5% of the human cells were CD34+ and 1.0% (~40% of the CD34+ human cells) were also positive for human Thy-1 (panels B and C). Of the 33 mice analyzed, all had detectable CD34+ cells in the BM (mean, 1.8 × 10⁴; range, 7.1 × 10² to 5.4 × 10⁵ per four leg bones), and 16 of 33 (48%) had detectable numbers of more primitive CD34+Thy-1+ cells (mean, 1.4 × 10⁴; range, 8.7 × 10² to 4.2 × 10⁴ per four leg bones).

To determine the numbers of human CFC present, unseparated BM cells or FACS-sorted human CD34+ cells were plated in methylcellulose under human-specific or standard conditions, respectively. CFC were detected in 37 of 45 (82%) mice analyzed. All mice that had detectable human cell engraftment (>0.1% human cells) also contained multiple types of lineage-restricted CFC, as well as CFC that generated multilineage colonies. Human CD34+ cells isolated from 31 mice were assayed for LTC-IC by using a 6-week CFC readout after maintenance of the cells on human growth factor (Steel factor, IL-3, and G-CSF)-producing murine fibroblasts.32 Human LTC-IC were detected in 16 of these mice.

Both normal and leukemic cells engraft the BM of NOD/SCID mice transplanted with CML cells. We previously found that the majority of human progenitors present in the BM of SCID mice that have been engrafted with PB or BM cells from patients with chronic phase CML were not leukemic.18 A similar analysis was therefore undertaken in the present study. From a total of 17 mice, 109 colonies were obtained and analyzed cytogenetically and 69 of these (71% ± 8%, Table 2) were found to contain the Ph chromosome. However, there was significant inter-mouse variability in the proportion of human progenitors present that were leukemic. In the one mouse where metaphases could be obtained from human LTC-IC–derived colonies, 15 of 17 were normal but the other 2 were Ph+(Table 2).

The proportion of leukemic (BCR+) cells among all the human cells present in the BM of the NOD/SCID mice transplanted with CML cells, as determined by Southern blotting using a human-specific 5' BCR probe, was found to range from 8% to 100% (66% ± 7%, mean ± SEM, n = 24 mice). A representative blot is shown in Fig 3. Interestingly, in

Fig 2. Detection by flow cytometry of human CD34-Thy-1+ cells in the BM of transplanted mice. Analysis of a representative mouse (18.1) transplanted with 10⁸ BM cells from patient 10 and killed 1 month later. (A) Histogram showing the proportion of viable (PI-) human (CD45/71+) cells present in the BM of this mouse. (B) and (C) Percentage of CD34+ and CD34-Thy-1+ cells determined by staining with human-specific anti-CD34-FITC and anti-Thy-1-phycocerythrin antibodies. Gates defining positive cells were set to exclude greater than 99.9% of cells stained with monoclonal antibodies of the same isotype and labeled with the corresponding fluorochromes.18,23
the blot of the BM from one of the mice shown, extra bands were seen, suggesting the presence of additional chromosomal rearrangements. However, this could not be definitively established by digestion of the same sample with a different restriction enzyme because of the limited quantity of DNA available. The limit of sensitivity of this Southern blotting procedure seemed to be 5%, because the germline band was generally not detectable if the level of human cell engraftment was below this value. Overall, there was no apparent correlation between the level of Ph\textsuperscript{1} cells and the proportion of human cells in the BM of the mice, the source (PB \, v \, BM) of cells transplanted, or whether or not human growth factors were injected. Two mice transplanted with 10\textsuperscript{8} PB or BM cells had high levels of engraftment (30% to 40%) of human cells in the spleen and Southern analysis of the DNA obtained from this site showed 4% and 10% BCR\textsuperscript{+} cells, respectively (data not shown).

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Cell Source</th>
<th>Time of Analysis (d)</th>
<th>No. of Mice</th>
<th>No. of Ph\textsuperscript{1} Colonies/ No. of Colonies Analyzed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>BM</td>
<td>14</td>
<td>1</td>
<td>1/1 (100)</td>
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<tr>
<td></td>
<td>PB</td>
<td>17</td>
<td>1</td>
<td>4/4 (100)</td>
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<td>4</td>
<td>BM</td>
<td>19</td>
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<td>20/25 (80)</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>10</td>
<td>1</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>5</td>
<td>PB\textsuperscript{*}</td>
<td>33</td>
<td>1</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>6</td>
<td>BM</td>
<td>16</td>
<td>1</td>
<td>10/16 (62.5)</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>21</td>
<td>1</td>
<td>3/8 (37.5)</td>
</tr>
<tr>
<td>7</td>
<td>PB</td>
<td>46-50</td>
<td>5</td>
<td>6/10 (60)</td>
</tr>
<tr>
<td>10</td>
<td>BM</td>
<td>29-51</td>
<td>5</td>
<td>18/35 (51.4)</td>
</tr>
</tbody>
</table>

Mean ± SEM 71 ± 8%

\textsuperscript{*}Human LTC-IC from this mouse were also analyzed and found to be predominantly normal (15 of 17 CFC progeny generated cytogenetically normal colonies); however, 2 of 17 were Ph\textsuperscript{1}.

*Human LTC-IC from this mouse were also analyzed and found to be predominantly normal (15 of 17 CFC progeny generated cytogenetically normal colonies); however, 2 of 17 were Ph\textsuperscript{1}.

Fig 3. Detection of normal and leukemic human cells in the BM of engrafted mice. Southern analysis of DNA extracted from the BM of NOD/SCID mice transplanted with 10\textsuperscript{8} PB cells from patient 6 and assessed 3 to 6 weeks later. Each numbered lane contains the DNA from a separate mouse. The DNA was digested with Bgl II and then probed using a Bgl II/HindIII fragment containing BCR exon 1\textsuperscript{31} to detect germline and rearranged BCR genes. (M) indicates the molecular weight marker. (P) indicates DNA extracted from the PB of the patient. The level of human cell engraftment in the BM of each mouse was determined separately by Southern blot analysis using a human-specific probe as described in Fig 1A. The percentage of BCR\textsuperscript{+} cells was determined as described in Materials and Methods. (ND), not detected.
For a more direct comparison, cells from an additional patient with chronic phase CML were transplanted into mice of both strains. One NOD/SCID mouse transplanted with $10^7$ unseparated BM cells was killed after 18 days because it appeared to be sick. Very high numbers of human myeloerythroid CFC were found in the murine BM, as well as a small number of multilineage CFC. Human CD34$^+$ cells and LTC-IC were also detected. A second NOD/SCID mouse transplanted with $1.5 \times 10^6$ enriched CD34$^+$ cells from the same donor and killed after 14 days had similarly high numbers of human CFC in the BM. Flow cytometric and LTC studies were not performed in this mouse.

In contrast, two SCID mice transplanted with $10 \times 10^7$ unseparated cells remained healthy and had 30- to 60-fold fewer human CFC in the BM after 6 weeks. No human CD34$^+$ cells were detectable. The vast majority (>90%) of human progenitors present in the BM of both NOD/SCID mice were Ph$^+$ by cytogenetic analysis.

**DISCUSSION**

In this study we describe an experimental in vivo model of human CML which involves the intravenous injection of patient PB or BM cells into sublethally irradiated NOD/SCID mice. The BM of these mice was routinely found to contain at least 1% human cells for up to 6.5 months after transplantation of $10^8$ light density cells from 10 of the 11 high WBC count chronic phase patients studied. Although the highest levels of human cells (up to 80%) were detected within 7 weeks posttransplant, the detection of human cells at the 1% to 10% levels for up to 6.5 months indicates the graft persists for long periods of time. Similar engraftment kinetics have recently been reported for normal human BM. CML PB cells engrafted as well as, or
only 17% of CFC were Ph1 in NOD/SCID recipients compared with SCID mice, in which CML cells made up a much larger proportion of the human graft previously using SCID hosts.18 For example, 25% of NOD/SCID recipients were much higher than those noted different patients, the levels of engraftment seen here in recipients of the same cells and between recipients of cells from (P = .015). The raw data for the SCID mice can be found in the report of Sirard et al.18

**Fig 5.** Comparison of engraftment of chronic phase CML cells in NOD/SCID versus SCID mice. Each (•) represents the mean level of human cell engraftment obtained after transplantation of PB or BM cells from individual patients with chronic phase CML into groups of NOD/SCID or SCID mice. Engraftment in NOD/SCID mice was significantly higher than in SCID mice (P = .015). The raw data for the SCID mice can be found in the report of Sirard et al.18

better than, BM cells, as was noted for similarly transplanted SCID mice.18 Although marked variability (>10-fold) in levels of engraftment were again seen, both between individual recipients of the same cells and between recipients of cells from different patients, the levels of engraftment seen here in NOD/SCID recipients were much higher than those noted previously using SCID hosts.18 For example, 25% of NOD/SCID recipients had 40% to 80% human cells, whereas only 3% of SCID recipients contained similarly high levels. Intermediate stages of human hematopoietic cell development detectable as in vitro CFC were found in a similar proportion of NOD/SCID and SCID recipients of CML cells (82% ± 77%), but more primitive cell types were found more frequently in the NOD/SCID mice (LTC-IC, 52% ± 13%; CD34+ cells, 100% ± 44%; CD34+Thy-1+ cells, 48% ± 0%).

The NOD/SCID mice were repopulated with both normal and leukemic human cells after transplantation of either PB or BM from patients with chronic phase disease. This was shown by analysis of colonies derived from both unsorted and purified human CD34+ CFC obtained from the BM of engrafted mice (71% Ph1 CFC), as well as by Southern analysis of the total human cell population present (up to 99% bcr rearranged). The CML cells made up a much larger proportion of the human graft in NOD/SCID recipients compared with SCID mice, in which only 17% of CFC were Ph1 and the lower engraftment levels precluded Southern analysis. These findings both confirm that primitive normal and leukemic cells are present in the PB and BM of patients with chronic phase CML at diagnosis,33 and that at least some of these have in vivo repopulating ability.18 The higher levels of engraftment that seem to be obtained in NOD/SCID mice suggest that this system may be further developed to provide an assay for these in vivo repopulating cells from patients with CML to allow their quantitation, as well as their further phenotypic and functional characterization. It is interesting to note that the predominance of genotypically normal human cells that was seen in similarly transplanted SCID mice18 was reversed in the NOD/SCID mice studied here. This suggests that the added immunodeficiency of the NOD/SCID strain, which includes deficient NK cell and macrophage function, may be more important for promoting the engraftment of the leukemic cells than of their normal counterparts, and that there is evidence that primitive Ph1 cells may be more sensitive to NK cells.34,35 However, these differences may also simply reflect interpatient variability in the relative and absolute prevalence of primitive normal and leukemic cells in their PB or BM. Thus, paired studies of cells from the same patients will be required to definitively resolve this question.

One of the hallmarks of CML is the inevitable progression of the disease to an acute leukemia. CML thus provides an important system in which to study the genetic changes that cause this to occur. As was seen in SCID mice,18 transplantation of blast crisis CML cells from one patient into NOD/SCID mice resulted in the rapid and extensive engraftment amplification of a leukemic population (data not shown). We previously reported that 100% of CFC derived from the BM of SCID mice transplanted with blast crisis cells were Ph1.18 Analysis of the total population of human cells present in the BM of the NOD/SCID recipients of blast crisis cells studied here also indicated that a high proportion were leukemic. It is also interesting that in one mouse transplanted with chronic phase cells (Fig 3, lane 2), extra bands were seen on BCR Southern analysis, suggesting either the outgrowth of a pre-existing subclone that was not detectable in the patient, or possibly that additional rearrangements had occurred in vivo posttransplant. Although such events would likely be rare, this model provides an in vivo system in which to analyze particular mutations that may contribute to leukemic progression, as the patterns of engraftment after transplantation of chronic phase or blast crisis cells seem to be distinct. For example, it might be possible to evaluate the role of various oncogenes in the progression of CML by transfecting them into chronic phase cells and then studying the engraftment potential of the transduced cells after transplantation into NOD/SCID mice.

The ability of NOD/SCID mice to be engrafted with leukemic cells after the transplantation of enriched populations of CD34+ cells from patients with chronic phase CML represents a first step towards determining the phenotype of CML stem cells. Previous studies of leukemic (Ph1/BCR1) LTC-IC have suggested that the CD34+HLA-DR– fraction of CML PB and BM is preferentially enriched for genotypically normal LTC-IC,36,38 whereas CD71, CD38, and Thy-1 are not useful markers for discriminating between Ph1 and Ph1– LTC-IC.33,37 The ability to detect engrafting cells capable of initiating human CML in NOD/SCID mice after transplantation of purified cells now provides a means to test whether the phenotypic properties of leukemic LTC-IC extend to CML cells with engrafting potential. In addition, CML-engrafted NOD/SCID mice should offer new opportunities for devising and testing novel therapeutic strategies.
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REFERENCES

34. Hauch M, Gazzola MV, Small T, Bordignon C, Barnett L, Cunningham I, Castro-Malaspina H, O’Reilly RJ, Keever CA: Anti-


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