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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CHRONIC MYELOID LEUKEMIA (CML) is a clonal multiligneage myeloproliferative disorder. It is now defined by the presence in the clonal cells of a unique genetic abnormality. In most cases, this is caused by a characteristic reciprocal exchange of genetic material, manifested cytogenetically as the Philadelphia chromosome (Ph).1 Involving the translocation of the c-abl proto-oncogene from chromosome 9 to a new position on chromosome 22 within the BCR-1 gene. This BCR-ABL fusion gene encodes a 210-kD chimeric protein product which has increased tyrosine kinase activity compared with the normal c-abl protein.6 Its presence in the cytoplasm is also believed to be key to its transforming activity. CML follows a biphasic or triphasic clinical course: an initial chronic phase characterized by the presence of a dominant clone of BCR-ABL^+/Ph^- cells which differentiate normally, followed inevitably a few years later by a blastic phase resembling acute leukemia.8 In some patients, the emergence of the blastic phase may be preceded by clinical signs of disease progression, referred to as an accelerated phase.

Much of our understanding of the biological changes underlying the pathophysiology of chronic phase CML has come from in vitro studies of the properties and behavior of lineage-restricted and multipotent colony-forming cells (CFC).7 More recently, long-term culture-initiating cells (LTC-IC) present in the peripheral blood (PB) and bone marrow (BM) of patients with CML.8 Early studies of long-term marrow cultures of cells from patients with CML together with clinical experience demonstrating the ability of patients to achieve cytogenetic remissions after treatment with high-dose chemotherapy9,10 or interferon-α11 showed the common persistence of a suppressed but functionally intact reservoir of normal (Ph^-) stem cells despite dominance of the Ph^+ clone in the CFC and more mature compartments of PB and BM cells. Ph^- LTC-IC have also been shown, but conditions that would allow the sustained growth of leukemic cells from either the PB or BM of patients with CML have not yet been identified.12-14 It has thus been difficult to investigate mechanisms underlying the in vivo expansion of the most primitive neoplastic (Ph^+) cells, or the role that p210BCR-ABL plays in initiating the disease.

Early attempts by other groups to establish an in vivo transplantation model of chronic phase CML by using severe combined immunodeficient (SCID) mice as recipients of human cells were not successful.15,16 Even blast crisis CML cells, when injected into SCID mice intraperitoneally, under the renal capsule, or into subcutaneously implanted human fetal bone fragments, were found to disseminate poorly to the BM of the mice.15-17 More recently, we reported that reproducible and sustained engraftment of SCID mice with intravenously transplanted chronic phase cells could be obtained if large enough numbers of cells from patients with high white blood cell (WBC) counts were injected and the mice were conditioned by a near-lethal dose of irradiation.18 Based on our prior success with this approach using transplants of normal human adult BM
or umbilical cord blood cells.\textsuperscript{19,20} 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,\textsuperscript{21} allow superior engraftment of normal\textsuperscript{22-24} and leukemic\textsuperscript{25} 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\textsuperscript+ cell–enriched populations. These experiments provide a foundation for the future characterization of the phenotype and properties of normal and Ph\textsuperscript+ cells that have long-term in vivo repopulating activity, as well as for the development of strategies to selectively manipulate normal and Ph\textsuperscript+ 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\textsuperscript+ 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\textsuperscript+ 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 Dinesh Jacob, Quantum Biosystems, Cambridge, UK), and anti-mouse Ig-coated magnetic beads (Dynabeads M-450, Dynal, Great Neck, NY) as described previously.\textsuperscript{26-28} CD34\textsuperscript+ cells were detached from the beads with Pasteurella haemolytica glycoprotease (Cedarlane, Hornby, Ontario, Canada) under conditions that efficiently removed 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\textsuperscript+ cell–enriched populations. These experiments provide a foundation for the future characterization of the phenotype and properties of normal and Ph\textsuperscript+ cells that have long-term in vivo repopulating activity, as well as for the development of strategies to selectively manipulate normal and Ph\textsuperscript+ stem cell populations in vivo.

| Table 1. Clinical Data for Patients’ Samples Used in This Study |
|-----------------|-----------------|-----------------|
| Patient No.*    | Age  | Sex | PB WBC Count (<10%) | Prior Treatment |
| 2               | 26   | M   | 395               | None           |
| 3               | 26   | M   | 45               | Hydroxyurea × 2 mos |
| 4               | 63   | M   | 58               | None           |
| 5               | 50   | F   | 160              | None           |
| 6               | 48   | M   | 135              | None           |
| 7               | 54   | M   | 132              | None           |
| 10              | 51   | F   | 135              | None           |
| 12              | 67   | F   | 189              | None           |
| 13              | 72   | M   | 218              | None           |
| 14              | 63   | M   | 123              | None           |
| 15              | 37   | M   | 82               | Hydroxyurea × 1 wk |
| 16              | 34   | F   | 120              | None           |
| 17              | 63   | M   | 87               | One 4-wk course of busulphan |

Abbreviations: M, male; F, female.

*All patients had Ph\textsuperscript+ 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 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 BglIII probe that contains the human BCR exon 1.\textsuperscript{31} 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; Labticonics, Inc, Guelph, Ontario, Canada). Results are expressed as mean ± SEM.

**RESULTS**

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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 all 65 mice transplanted with chronic phase CML cells, as determined by Southern blot analysis (+ and ●) or flow cytometry (▲ and ○) using antibodies to CD45 and CD71. The number of cells transplanted per mouse ranged from 7.5 × 10^7 to 10^8 except for two mice that received 2 × 10^7 PB cells and two mice that received 8 × 10^6 PB cells. (—) Indicate geometric means.

mice (25%) for up to 7 weeks posttransplant. All 9 of 9 mice that were analyzed between 3 and 6.5 months posttransplant were engrafted and 6 of these mice contained between 1% to 10% human cells, indicating that the graft persists for a long period of time. Human cell engraftment (>1% human cells) was observed in mice transplanted with cells from 10 of the 11 patients. The degree of engraftment tended to be higher after transplantation of the same number of PB as compared with BM cells. (The BM of all 39 mice transplanted with CML PB cells contained at least 1% human cells, whereas this was true for only 18 of 26 mice (69%) transplanted with CML BM cells.) Treatment of transplanted mice with various human cytokines on alternate days did not obviously enhance the level of human cell engraftment (data not shown).
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 (PI−) 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 × 10^4; range, 7.1 × 10^2 to 5.4 × 10^5 per four leg bones), and 16 of 33 (48%) had detectable numbers of more primitive CD34+Thy-1+ cells (mean, 1.4 × 10^3; range, 8.7 × 10^2 to 4.2 × 10^4 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. 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. 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...
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 Ph1 cells and the proportion of human cells in the BM of the mice, the source (PB or BM) of cells transplanted, or whether or not human growth factors were injected. Two mice transplanted with 108 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+ cells, respectively (data not shown).

Table 2. Detection of Leukemic CFC in the BM of Mice Transplanted With Chronic Phase CML Cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Cell Source</th>
<th>Time of Analysis (d)</th>
<th>No. of Mice</th>
<th>Metaphase Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB</td>
<td>17</td>
<td>1</td>
<td>No. of Ph1 Colonies/No. of Colonies Analyzed (%)</td>
</tr>
<tr>
<td>2</td>
<td>BM</td>
<td>14</td>
<td>1</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>4</td>
<td>BM</td>
<td>19</td>
<td>1</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>5</td>
<td>PB*</td>
<td>33</td>
<td>1</td>
<td>20/25 (80)</td>
</tr>
<tr>
<td>6</td>
<td>BM</td>
<td>16</td>
<td>1</td>
<td>10/16 (62.5)</td>
</tr>
<tr>
<td>7</td>
<td>PB</td>
<td>21</td>
<td>1</td>
<td>3/8 (37.5)</td>
</tr>
<tr>
<td>8</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>
<tr>
<td><strong>Mean ± SEM</strong></td>
<td></td>
<td></td>
<td><strong>71 ± 8%</strong></td>
<td></td>
</tr>
</tbody>
</table>

*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 Ph1.

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 108 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 131 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+ 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
better than, BM cells, as was noted for similarly transplanted SCID mice. 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. 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% v 77%), but more primitive cell types were found more frequently in the NOD/SCID mice (LTC-IC, 52% v 13%; CD34+ cells, 100% v 44%; CD34+Thy-1+ cells, 48% v 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% Ph+ 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 Ph+ 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, and that at least some of these have in vivo repopulating ability. 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 mice 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 some evidence that primitive Ph+ cells may be more sensitive to NK cells. 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, 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 Ph+. 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 (Ph+/BCR+) LTC-IC have suggested that the CD34+ HLA-DR− fraction of CML PB and BM is preferentially enriched for genotypically normal LTC-IC, whereas CD71, CD38, and Thy-1 are not useful markers for discriminating between Ph+ and Ph− LTC-IC. 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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