Bone Marrow From Children in Relapse With Pre-B Acute Lymphoblastic Leukemia Proliferates and Disseminates Rapidly in scid Mice

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Bone marrow samples from patients with pre-B acute lymphoblastic leukemia (pre-B ALL), either at diagnosis or at relapse, were transplanted into scid mice to determine whether these freshly obtained leukemic cells could proliferate in vivo and whether there were any differences in their in vivo growth characteristics. Cells from three patients who relapsed within 13 months of diagnosis proliferated rapidly in the murine bone marrow, spleen, and thymus, invaded peripheral organs, and resulted in morbidity and mortality of the animals within 4 to 16 weeks. Cells from two patients who relapsed 3.5 years after diagnosis grew much slower than the early relapse samples, taking up to 30 weeks to infiltrate the bone marrow of recipient mice. In contrast, leukemic cells were absent or were detected at low numbers in scid mice transplanted with cells obtained at diagnosis from three patients who have not yet relapsed. These results show an increased ability of leukemic cells from patients with aggressive lymphoblastic leukemia of poor prognosis to proliferate in scid mice.

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RESULTS

Characteristics of patients with pre-B ALL at different stages of disease. The eight patients examined in this study were between 2 and 14 years of age and had either a group III or group IV pre-B ALL phenotype (Table 1). Three cases R-1, R-2, and R-3 relapsed within 13 months of diagnosis, R-4 and R-5 relapsed within 3 to 4 years, and D-1, D-2, and D-3 have not yet relapsed (Table 1). Clinically, it was not possible to establish a durable second remission in any of the early or late relapse patients except for R-5, who is in remission after a BM transplant. Because each of these groups of patients had a different clinical course and outcome of their disease, we wanted to determine whether any of these samples could grow in scid mice and whether there were any differences in their in vivo growth characteristics. Leukemic cells from these patients were transplanted into scid mice using our previously described procedures. Mice (the number of animals was dependent on the excess quantity of cells in the diagnostic aspirates) were irradiated with 400 cGy before intravenous injection of 3 to 10 × 10⁶ Percoll-enriched BM cells from each of these patients. Generally, mice were killed when they showed clinical signs of disease (weight loss, lethargy, ruffled fur). The presence of leukemic cells in the BM, spleen, and thymus of these animals and the extent of leukemic infiltration into non-hematopoietic tissues were measured by a combination of flow cytometry using markers of leukemic cells (CD10, CD44, HLA-DR, and CD19), DNA analysis using probes specific for the α-satellite sequences on human chromosome 17, and histochemical staining.

Transplantation of leukemic samples from early relapse patients into scid mice. The flow cytometry analysis, using CD10 and CD44, of the BM, spleen, and thymus of representative scid mice that received BM from cases R-1, R-2, and R-3 at relapse, is shown in Fig 1A and the data from all of the animals tested is summarized in Table 1. High levels of CD44+ leukemic cells were found in the BM and thymus of the mice killed 4, 11, and 18 weeks after injection of R-1, R-2, and R-3 cells, respectively; high levels were also seen in the spleen of animals injected with cells from R-1 and R-2, but lower levels were seen for R-3. The level of CD10+ was lower than CD44+ on cells for R-1 and R-3, which reflects the phenotype of the original patient samples. It was interesting that the small thymus found in scid mice provided a suitable location for these leukemic cells to proliferate. The presence of leukemic cells in the hematopoietic tissues and in the lung, liver, kidney, and brain of transplanted mice was determined by DNA analysis using human-specific probes. Comparison of the intensity of the 2.7-kb EcoRI band, characteristic of the α-satellite sequences on human chromosome 17, between the known human:mouse control mixtures and the scid samples showed heavy infiltrates of human cells in all of the tissues tested, indicative of aggressive, widely disseminated leukemia (Fig 1B; Table 1). Although the BM from R-3 took longer (18 weeks) than R-1 (4 weeks) and R-2 (11 weeks) to grow in the mice, it should be noted that the BM sample had been frozen and the actual number of viable cells injected into mice was 3 × 10⁶. To determine whether the human leukemic cells could be passaged in scid mice and whether their growth characteristics would change, BM (10⁶ cells) from R-1 was serially
Table 1. Characteristics of Patients With Pre-B ALL and Growth Patterns of Their Leukemic Cells in scid Mice

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>FAB/Pre-B Class*</th>
<th>Time From Diagnosis</th>
<th>Leukemic Blasts in Injected Sample (%)</th>
<th>Time to Obtain Cell Line</th>
<th>Growth in SCID Mice (Longest Time of Observation/Extent of BM Infiltrate)$</th>
<th>No. of Mice With Leukemic Cells/Total No. Injected</th>
<th>Summary of DNA and Histology Analysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kidney DNA</td>
</tr>
<tr>
<td>R-1</td>
<td>2</td>
<td>F</td>
<td>L1/IV</td>
<td>Relapse-1: 13 mo</td>
<td>87</td>
<td>8 wk</td>
<td>6wk/+++</td>
<td>41/41</td>
<td>+++</td>
</tr>
<tr>
<td>R-2</td>
<td>14</td>
<td>F</td>
<td>L2/IV</td>
<td>Relapse-1: 10 mo</td>
<td>93</td>
<td>10 wk</td>
<td>12wk/+++</td>
<td>6/6</td>
<td>+++</td>
</tr>
<tr>
<td>R-3</td>
<td>14</td>
<td>M</td>
<td>L1/IV</td>
<td>Relapse-15: 4 mo</td>
<td>95</td>
<td>8 wk</td>
<td>4mo/+++</td>
<td>1/1</td>
<td>ND</td>
</tr>
<tr>
<td>R-4</td>
<td>11</td>
<td>M</td>
<td>L1/III</td>
<td>Chemo for 36 mo</td>
<td>94</td>
<td>No line</td>
<td>6mo/+++</td>
<td>5/7</td>
<td>+</td>
</tr>
<tr>
<td>R-5</td>
<td>10</td>
<td>M</td>
<td>L1/IV</td>
<td>Chemo for 36 mo</td>
<td>13</td>
<td>No line</td>
<td>8mo/-</td>
<td>4/5</td>
<td>+</td>
</tr>
<tr>
<td>D-1</td>
<td>1</td>
<td>M</td>
<td>L1/IV</td>
<td>Diagnosis§</td>
<td>95</td>
<td>No line</td>
<td>6mo/-</td>
<td>4/4</td>
<td>+</td>
</tr>
<tr>
<td>D-2</td>
<td>2</td>
<td>F</td>
<td>L1/III</td>
<td>Diagnosis§</td>
<td>90</td>
<td>No line</td>
<td>9mo/-</td>
<td>4/9</td>
<td>+</td>
</tr>
<tr>
<td>D-3</td>
<td>6</td>
<td>F</td>
<td>L1/III</td>
<td>Diagnosis§</td>
<td>96</td>
<td>No line</td>
<td>4mo/-</td>
<td>1/2</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

*French-American-British classification of leukemic morphology: pre-B ALL class III defined as HLA-DR+, CD19+, CD10+, CD20-; and class IV defined as HLA-DR+, CD19+, CD10-, CD20-.†

†Leukemic blasts in BM at times indicated as measured by flow cytometry (−, no cells detected; +, 1% to 5%; ++, 5% to 50%; ++++, 50% to 100% human leukemic cells).

‡The extent of leukemic cell infiltration scored by hematoxylin and eosin-stained sections from mice analyzed at the times indicated: −, no cells detected; +, occasional leukemic cells; ++, focal aggregates of leukemic cells; ++++, wide-spread infiltrates; and by DNA: −, no human DNA detected; +, 0.1% to 1.0%; ++, 1.0% to 10%; ++++, 10% to 100% human DNA.

§Sample injected into scid mice.

||Includes mice with small leukemic infiltrates at 4 to 6 week time points only.
Fig 1. Analysis of tissues from scid mice injected with BM cells taken, at relapse, from pre-B ALL patients R-1, R-2, and R-3, whose disease recurred within 13 months of diagnosis. The injected samples were either freshly obtained (R-2), frozen (R-3), or BM from a primary animal transplanted with R-1 cells. BM from mice transplanted with R-1 cells was serially passaged every 6 weeks up to eight times; all mice showed the same profiles of leukemic infiltration as the secondary animal shown here. (A) Flow cytometry analysis of the BM, spleen, and thymus. Cell suspensions from the BM, spleen (S), and thymus (Th) were stained with MoAb 44ClO (antihuman CALLA/CD10) or with MoAb 5084 (antihuman CD44) and with FITC-goat F(ab)\textsubscript{2} antimouse IgG and analyzed by flow cytometry. The percent cells positive as determined by reference to the appropriate isotype control is indicated on each histogram. Profiles are representative of animals killed either at 4 weeks (R-l), 11 weeks (R-2), or 18 weeks (R-3). (B) DNA analysis of the BM, spleen (SP), lung (LU), liver (LIV), kidney (KID), brain (BR), or thymus (THY) of the same scid mice shown above. In addition, DNA was analyzed from R-1 mice 2, 4, and 5 weeks after injection. DNA (5 pg) was digested with EcoRI, blotted according to standard protocol, and probed with p17H8, a human a-satellite probe specific for human chromosome 17 sequences. The percentage of human DNA present in the mouse tissues was estimated using the human:mouse mixtures indicated: 0% human:100% mouse DNA; 0.1% human:99.9% mouse DNA; 1% human:99% mouse DNA; 10% human:90% mouse DNA. The autoradiograph for samples from mice transplanted with R-1 cells and the adjacent controls was exposed for 8 hours while the other samples and adjacent controls were exposed for 4 hours after using probes with different specific activities.

Histologic examination of the tissues obtained from the mice engrafted with each of the relapse samples was consistent with aggressive leukemic proliferation (Fig 2; Table 1). Figure 2 shows a representative mouse engrafted with cells from patient R-1. The kidneys showed extensive leukemic cell infiltration of the interstitial regions under the capsule and in the perirenal fat. In the liver, leukemic cells extensively infiltrated into the periportal region and around the central vein extending into the hepatic sinusoids. The normal kidney and liver morphology was obliterated by the presence of blasts in some areas. Leukemic cells also infiltrated the cerebral cortex within the meningeal

passaged in scid mice every 6 weeks when the animals showed clinical signs of disease. We passaged the cells eight times with no noticeable changes in the growth rate, extent, or pattern of leukemic infiltration between passages 2 and 8, indicating that in vivo passage was not selecting for faster growing variants. Kinetic studies at the second passage showed the rapid proliferation of cells initially in the BM with heavy infiltrates detected as early as 2 weeks (Fig 1B). Dissemination of leukemic cells occurred within the next 2 weeks into spleen, kidney, lung, liver, thymus, and, to a lesser extent, brain. At 5 weeks, each of these organs was heavily infiltrated and the animals died by week 6.
space. The lungs also showed heavy infiltrates (data not shown).

In addition to transplantation into scid mice, the BM samples from these relapse patients were also grown in vitro culture using previously described methods.25 Cell lines were established within 8 to 10 weeks (Table 1). The in vitro and in vivo growth characteristics of a line derived from R-1 will be published elsewhere.26

Transplantation of leukemic samples from late relapse patients into scid mice. BM samples obtained at relapse from cases R-4 and R-5, who both relapsed 3 to 4 years after diagnosis, grew more slowly in scid mice than the early relapse samples (Fig 3A and B; Table 1). Scid mice injected with cells from R-4 were killed at 4, 8, and 27 weeks. We previously showed that the BM from scid mice transplanted with R-4 cells contained 1% to 10% leukemic cells at 4 and 8 weeks (patient 2 in Kamel-Reid et al1a). Two additional animals were followed-up for 27 weeks and killed when they began to show signs of disease. One of the animals (not shown) had a thymoma of mouse origin and no human cells were detected in any tissues by DNA analysis (not shown). The BM of the other animal (R-4-1a) contained 78% CD10+ and the spleen 6% HLA-DR+ leukemic cells (Fig 3A). DNA analysis on the tissues of this mouse showed a variable degree of infiltration, with the lung and liver showing the highest level of infiltration (5% to 10%) (Fig 3B). The low number of BM cells precluded DNA analysis. BM passaged from this mouse into another scid mouse (R-4-2a) again took 30 weeks to reach similar levels of infiltration, indicating that in vivo passage was not selecting for faster growing variants (Fig 3A and B).

Mice injected with BM from case R-5, who relapsed 42 months after diagnosis, were killed 8 months later; the level of engraftment, as determined by flow cytometry, was low, with 6% leukemic cells detected in the BM (Fig 3A). DNA analysis substantiated this level of engraftment in the BM; small infiltrates of human cells were also detected in the kidney, liver, lung, brain, and thymus, while no human DNA was detected in the spleen. Histologic examination of tissues from these mice confirmed the presence of small foci of leukemic cells (not shown). These data indicate that R-4 cells were more aggressive in their growth pattern in scid mice than R-5 cells. However, it is also possible that the lower level of proliferation was due to the fact that the BM sample from R-5 contained fewer leukemic blasts, resulting in less leukemic cells being transplanted into scid mice (Table 1). The clinical outcome for R-5 and R-4 was also different. It was possible to establish a second remission in R-5; the patient had a BM transplant 15 months ago and is still disease free. A durable second remission could not be established in R-4.

Transplantation of leukemic samples from patients at diagnosis into scid mice. In contrast to the results obtained with cells from patients at relapse, the BM samples taken from patients at diagnosis did not proliferate or else proliferated to low levels after injection into scid mice (Table 1). Flow cytometry analysis showed the presence of 5% human leukemic cells in the BM of scid mice 5 months after engraftment with BM from case D-1 (Fig 4A). DNA and histologic analyses indicated that other tissues were either uninvolved or infiltrated at a low level (1%) (Fig 4B; Table 1). We have previously shown low levels of human cells in scid mice 4 to 10 weeks after transplantation with diagnosis samples from D-2 and D-3 (patient 1 and 3 in Kamel-Reid et al1a); however, no human cells could be detected by flow cytometry, DNA, and histochemical analysis of additional mice from these groups after longer periods of observation (up to 9 months) (Table 1). None of the patients whose diagnostic sample failed to grow to a significant extent in scid mice have relapsed to date.

DISCUSSION

The experiments described here establish that leukemic cells from patients with poor prognosis, ie, those who

Fig 2. Histologic analysis of the kidney, liver, and brain of a scid mouse transplanted with leukemic cells from patient R-1 at 5 weeks after the second passage. Tissues were fixed in 10% formalin, paraffin-embedded, and 4 μm sections were cut and stained with hematoxylin and eosin. (A) Section of the kidney with leukemic infiltration in the interstitium. (B) Section of the liver showing heavy leukemic infiltrates around the periportal region extending into the hepatic sinusoids. (C) Section of cerebral cortex showing a dense infiltration of the meninges by leukemic cells. Original magnification × 300. The results are representative of all the mice engrafted with leukemic cells from early relapse patients.
relapse less than 13 months after diagnosis while still on chemotherapy, grew rapidly in scid mice and disseminated to many peripheral organs, including the brain. The dissemination of leukemic cells into peripheral organs such as the brain is frequently observed in children with advanced disease. The cell surface phenotype of the leukemic cells growing in scid mice was the same as the original transplanted population. Samples obtained from patients who relapsed late after diagnosis took much longer to proliferate in the BM and spleen of scid mice and the extent of dissemination to peripheral organs was lower than for the poor prognosis patients. In contrast, samples taken from patients at diagnosis who have not relapsed to date (18 to 28 month follow-up) grew little, if at all.

Pre-B ALL cells have complex growth factor requirements for in vitro growth and typically need to be cultured at high cell density in autologous conditioned medium and/or recombinant growth factors to establish cell lines. The extensive proliferation of human pre-B ALL cells from patients at relapse in scid mice suggests that the murine microenvironment can support the growth of leukemic cells, perhaps by supplying a critical growth factor. This
idea is strengthened by experiments that showed that some factor-dependent pre-B ALL cells could be supported on adherent layers of murine stromal cells. There was an absolute requirement for cell-cell contact because the leukemic cells did not proliferate in stromal cell-conditioned medium. We have similarly found that the human growth factor-dependent myeloid cell line Mo7E can proliferate in immune-deficient mice as well as on murine embryo fibroblasts (unpublished).

Cell lines were established from the patients with aggressive clinical disease (R-2, R-1, and R-3) using previously reported techniques including incubation in various combinations of recombinant growth factors as well as autologous conditioned media prepared from the leukemic cells themselves (Table 1). However, no cell lines could be established from any other patient samples, nor from the heavily infiltrated tissues of scid mice transplanted with R-4 cells. Despite the slow growth of R-4 in primary scid mice, they could be passaged into secondary scid recipients. Therefore, the proliferation of human leukemic cells in vivo provides a valuable method to expand and preserve leukemic cells that cannot be established in culture.

After an initial response to chemotherapy, approximately 20% of children with pre-B ALL have recurrence of leukemia that is often drug-resistant and difficult to treat effectively; these children have a much poorer prognosis than those with newly diagnosed leukemia. The growth of drug-resistant relapse leukemic samples in scid mice provides a model to test treatment modalities directed against the drug-resistant cells that are probably more relevant than in vitro drug studies. In addition, several approaches have been developed to detect small numbers of residual leukemic cells either as prognostic tools or to test the efficacy of BM purging techniques. These approaches include clonogenic assays for the leukemic stem cell or the molecular detection of specific chromosomal abnormalities by polymerase chain reaction (PCR). The advantage of the scid model over molecular detection methods such as PCR is that it provides a biologic assay for residual leukemia by directly testing the ability of the cells to grow...
and disseminate. The experiments in this report show that the transplantation of between 3 and 10 × 10^6 leukemic cells from relapse patients into scid mice leads to leukemic proliferation. A large limiting dilution experiment was performed using a cell line (G2) derived from patient R-1. We have found that as few as 100 G2 cells were sufficient to cause leukemic proliferation in scid mice, suggesting that the scid model can support the growth of low numbers of aggressive leukemic cells. It will now be interesting to follow-up a larger group of patients, from diagnosis to relapse, to determine whether low numbers of residual leukemic cells in patients who relapse can be detected in scid mice. The low frequency of relapse cases in pre-B ALL will make it necessary initially to enroll a large number of patients and observe them for a long period of time. Our finding that frozen cells can be transplanted into mice should simplify the design of such a large study.

REFERENCES


Bone marrow from children in relapse with pre-B acute lymphoblastic leukemia proliferates and disseminates rapidly in scid mice

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