An Approach for the Analysis of Relapse and Marrow Reconstitution After Autologous Marrow Transplantation Using Retrovirus-Mediated Gene Transfer


Autologous bone marrow transplantation (ABMT) is widely used as treatment for malignant disease. Although the major cause of treatment failure is relapse, it is unknown if this arises entirely because of residual disease in the patient or whether contaminating cells in the rescuing marrow contribute. Attempts to purge marrow of its putative residual malignant cells may delay hematopoietic reconstitution and are of uncertain efficacy. We now describe how retroviruses-mediated gene transfer may be used to elucidate the source of relapse after ABMT for acute myeloid leukemia and to evaluate the efficacy of purging. Clonogenic myeloid leukemic blast cells in patient marrow can be transduced with the Neo<sup>+</sup> gene-containing helper-free retrovirus, LNL6, with an efficacy of 0% to 23.5% (mean, 10.5%). Transduced colonies grow in selective media and the presence of the marker gene can be confirmed in individual malignant colonies by polymerase chain reaction. If such malignant cells remain in harvested “remission” marrow, they will therefore be marked after exposure to LNL6. Detection of the marker gene in the malignant cells present at any later relapse would be firm evidence that residual disease contributed to disease recurrence, and would permit rapid subsequent evaluation of purging techniques. The technique also marks normal marrow progenitors from patients with acute myeloblastic leukemia. These colony-forming cells can be detected in long-term marrow cultures at a frequency of 1% to 18% for up to 10 weeks after exposure to the vector. Animal models and analysis of probability tables both suggest that these levels of marking in vitro are sufficient to provide information about the mechanisms of relapse and the biology of marrow regeneration in vivo. These preclinical data form part of the basis for current clinical studies of gene transfer into marrow before ABMT.

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THE COMBINATION of supralethal chemo/radiotherapy and subsequent autologous bone marrow transplantation (ABMT) as hematopoietic rescue has proved effective in treating many malignancies, yet an unacceptable proportion of patients subsequently relapse. Because the rescue marrow is harvested when the patient is in clinical remission, before the administration of supralethal therapy, it is unknown whether relapses result from residual disease in the patient or from malignant cells contaminating the infused marrow. This is a crucial issue. Concern that harvested marrow may contain residual malignant cell has led to extensive evaluation of techniques to purge marrow before reinfusion. Despite promising animal and preclinical human data, none of these techniques have yet been shown to reduce the risk of relapse. Further, almost all damage normal progenitor cells, thus slowing engraftment and increasing early morbidity and mortality.

If harvested marrow were transduced with a marker gene and malignant cells carrying this marker were subsequently detected at relapse, this would be powerful evidence that the harvested marrow does contribute to relapse. Moreover, this finding would permit rapid subsequent evaluation of ex vivo purging techniques. In addition, if normal progenitor cells in remission marrow could be marked, then detection and assessment of the subsequent in vivo growth of these cells could provide information about the clonality and cytotoxic responsiveness of the infused marrow. This would allow the contribution of ABMT to hematopoietic recovery to be distinguished from repopulation attributable to residual endogenous marrow. The availability of this information would, in turn, facilitate the design of future therapeutic protocols aimed at transferring genes into long-lived marrow progenitor cells rather than fully differentiated lymphocytes.

We describe here successful gene transfer into normal and malignant marrow clonogenic progenitor cells from patients with active acute myeloblastic leukemia (AML), and transduction of normal progenitor cells from patients in remission after chemotherapy. The efficiency of transfer reached levels that should permit evaluation of the source of relapse after ABMT and clarify the mechanisms of post-ABMT hematopoietic recovery. These data have contributed to the design of ongoing clinical BM gene transfer protocols.

MATERIALS AND METHODS

Patient details. A total of 18 patients with AML were studied. All were treated on institutional protocols: 10 were studied in remission and eight at presentation or relapse. Seven normal marrow donors were also investigated. Approval was obtained for these studies from the St Jude Children’s Research Hospital Institutional Review Board and Biosafety Committee. All patients and normal donors or their legal guardians gave written informed consent.

Transduction with LNL6 and short-term marrow culture. After administration of local or general anesthesia, BM was withdrawn from the posterior iliac crest and collected in preservative-free sterile heparin. Mononuclear cells were prepared by Ficoll-Hypaque separation. After final washing, an aliquot of $5 \times 10^5$ cells was removed and resuspended in 1 mL of RPMI plus 10% fetal calf serum (FCS), 1% penicillin-streptomycin (a 10,000 U/10,000 µg/mL solution), and 1% L-glutamine. These marrow cells were cultured in 2.2% methylcellulose (Sigma, St Louis, MO) that was dissolved in α-MEM with 10% bovine serum albumin (BSA), 10% by volume of phytohemagglutinin-leukocyte conditioned medium.
(PHA-LCM), recombinant human erythropoietin (Amgen, Thousand Oaks, CA), 1 U/mL, 10^{-4} mol/L 2-mercaptoethanol, 10^{-6} mol/L methylprednisolone sodium succinate, and L-glutamine at 1% of a 200 mmol/L solution. Each 1 mL of methylcellulose with marrow cells was plated in 35 x 10 mm Petri dishes (Nunc, Naperville, IL) and incubated at 37°C in 5% CO2 in 80% humidity for 12 to 14 days. Cells were cultured with and without the neomycin analogue G418 at 1 mg/mL (active concentration) at a final cell concentration of 5 x 10^6/mL. G418 was prepared by dissolving the drug in water for injection and adding 10% of 1 mol/L HEPES tissue culture grade. The pH was adjusted to 7.2 to 7.4 with 10 N NaOH and the solution filter sterilized.

LN6 clinical grade virus conferring resistance to G418 was provided by Genetic Therapy Inc (Gaithersburg, MD) and was produced by using PA317 amphotropic retrovirus packaging cells containing the LN6 vector25 (PA317/LN6 c8). Virus was added to the remaining cells. It was used at multiplicity of infection (MOI) of 10. Protamine sulfate (preservative-free injection NDC 0641-2554-41; Elkins Sinn, Inc, Cherry Hill, NJ) was added at a concentration of 4 µg/mL.26

Cells and supernatant were incubated in T175 tissue culture flasks (Nunc) at 37°C with CO2 and 80% humidity for 6 hours and mixed gently every 2 hours. After incubation, cells were pelleted by spinning at 1,200 rpm for 10 minutes. The cells recovered were counted and placed in methylcellulose cultures with or without G418, and with and without PHA-conditioned media as a source of growth factors (see below). Plating concentrations were 2.5 x 10^6/mL for transduced marrow.

Colonies were identified and counted at 12 to 14 days on duplicate or triplicate plates with the results averaged. Colonies were defined as collections of ≥50 cells and characterized as Malignant (blast), burst-forming unit-erythroid (BFU-E), colony-forming unit-erythroid (CFU-E), CFU-granulocyte (CFU-G), CFU-granulocyte-macrophage (CFU-GM), CFU-macrophage (CFU-M), or CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) according to morphology, with confirmation on histochemical staining of selected individual colonies. Individual colonies were “picked” and processed for polymerase chain reaction (PCR) and morphologic/phenotypic analysis.

The efficiency of transduction was calculated as:

\[
\text{Efficiency} = \frac{\text{No. of Colonies Growing in the Absence of G418}}{\text{No. of Colonies Growing in the Presence of G418}} \times 100
\]

**RESULTS**

The numbers of normal precursor and malignant cell colonies formed in the presence of increasing concentrations of G418 are shown in Fig 1. In the absence of transduction, a 1.0 mg/mL (active) concentration of G418 consistently produced complete inhibition of colony growth, with survival seen only in transduced samples.

![Fig 1](https://www.bloodjournal.org/bjcurrent/pdf/75/21/2695b.jpg)
studied, all colonies picked from selective media contained the NEO-R gene. No significant difference was found between the rate of marking (percentage of NEO-R-positive colonies in nonselective media), which was 0% to 18% (mean, 12.5%), and the rate of NEO-R-expression (number of colonies growing in G418 versus the number growing in nonselective media), which was 0% to 23.5% (mean, 10.5%). Thus, in these short-term clonogenic assays, most NEO-R-transduced clonogenic cells also express the NEO-R gene.

Because the effects of the vector supernatant on the engraftment of marrow are unknown, only an aliquot of marrow (30%) will be exposed to the vector in clinical marrow-marking protocols. Given the finding that only about 10% of clonogenic AML cells are marked, approximately 3% of putative residual clonogenic malignant cells would be marker gene positive. We therefore constructed a probability table to determine the likelihood that a relapse containing marked cells would occur with this low an efficiency of marking. As shown in Table 1, a transduction efficiency of 3% yields a greater than 95% chance of detecting a marked relapse in any given case, provided that ≥ 100 cells contribute to relapse. Even with a transduction efficiency of 0.3%, one-tenth the level obtained, there is a 60% chance of detecting an individual relapse if 300 cells contribute, and a greater than 95% chance of detecting one marked relapse in a cohort of three patients.

Normal progenitor cells from patients whose AML is in remission can also be transduced. The numbers of myeloid, erythroid, and mixed colonies identified in cultures of marrows from 10 AML patients in remission 4 to 14 weeks postchemotherapy are shown in Fig 4A in comparison to the numbers of colonies observed in cultures from seven normal donors. At this stage postchemotherapy, the number of erythroid colonies is greater in the patient group than in the controls (P < .05). These normal progenitor cells from both groups could be transduced and will grow as G418-resistant colonies in 14-day selective cultures. Transduction rates were not significantly different in marrow obtained from normal donors or from AML patients in remission and ranged between 1.2% and 17.9%, with the highest rates obtained for late-erythroid (CFU-E) and granulocytic colonies, and the lowest for the mixed GEMM

Figure 2 shows the results of exposing marrow samples obtained at presentation or relapse of AML (n = 8) to the helper-virus-free LNL6 vector. In five of eight samples, colonies subsequently grew in methylcellulose cultures. The transduction rate assessed by NEO-R expression (ie, number of blast colonies in G418 containing cultures versus numbers in control media) ranged from 0% to 23.5% with a mean of 10.5%. Recovery rates (the number of malignant colonies growing in nonselective (control) media before and after transduction) were between 66% and 78%.

To confirm that G418 resistance was due to the presence of the vector-derived NEO-R gene and to determine whether gene transfer occurred at a significantly higher rate than gene expression,26 individual blast colonies were picked from selective (G418) and from control plates and examined by PCR for the 790-bp NEO-R-derived fragment. Results from a representative patient sample are shown in Fig 3; all blast colonies from selective media contain the NEO-R gene, whereas only one-fourth of those from control media are so marked. In all patient samples

![Image](https://www.bloodjournal.org/bcell/fig2.png)

**Fig 2.** Transfer and expression of NEO-R gene can be detected in G418-containing methylcellulose cultures. Transduced and nontransduced marrow from patients in presentation/relapse of AML were cultured with and without G418. The blast colonies growing in G418 as a percentage of the colonies growing in nonselective media are shown. Mean colony number in nonselective media, 117 ± 195 (SD); range, 4 to 488. Recovery rates (number of colonies after transduction in nonselective media/number of colonies without transduction in nonselective media × 100%) equals 66% to 78%.

![Image](https://www.bloodjournal.org/bcell/fig3.png)

**Fig 3.** Transfer efficiency assessed by PCR. Individual colonies were picked from nonselective (lanes 1 through 4) or selective media. The 790-bp neo<sup>+</sup> insert was identified using the primers and methodology described. There was no significant difference between transfer rates assessed by NEO-R expression or NEO-R content in these short-term cultures. Transfer rates assessed by PCR, 0% to 33%; mean ± SD, 12%. +, Plasmid control; −, nontransduced control.
precursors (Fig 4B). Individual colonies were identified and studied for the presence of the NEO-R gene using PCR and results from one remission patient are shown in Fig 5. In selective media all surviving colonies contained the NEO-R gene, while in control media only 2 of 20 picked colonies were NEO-R-positive.

Longer-lived precursor cells can also be transduced. Figure 6A through C illustrates the growth of G418-resistant colonies from long-term (Dexter-type) cultures of patient marrow cells transduced by LNL6. All precursor types except GEMM were detectable and were marked. The ratio of transduced:nontransduced colonies remained constant over 8 to 10 weeks before subsequent declining.

**DISCUSSION**

The studies described here show that both normal and malignant BM clonogenic cells from patients with AML can reproducibly be transduced, and that NEO-R-containing clones can be detected for up to 10 weeks in culture. These studies are preliminary to the first clinical protocol designed to transfer a marker gene into normal and malignant marrow cells. A significant proportion of the patients being enrolled on that study should have a normal life expectancy, so considerations of long-term safety are of paramount importance. Fortunately, for reasons extensively reviewed elsewhere,29 the major risks of transduction (insertional mutagenesis and viral recombination with endogenous retrovirus) are considered to be of very low probability. Our preclinical studies support this contention. We found no evidence of transformation, and the ratio of NEO-R-positive:NEO-R-negative clonogenic cells in long-term cultures remained constant over a 3-month period (Fig 6). Supernatants from transduced cultures had no reverse transcriptase or retrovirus activity by the S+L- or NIH-3T3-amplification assays (data not shown), so there was no evidence for contamination with helper virus or of recombination with human endogenous retrovirus sequences.

Although the risks of transducing marrow precursor cells with a marker gene appear to be small, they nonetheless exist. Therefore, gene marking of marrow can only be justified if it has a reasonable chance of answering important questions not amenable to study by other available technologies. These conditions appear to obtain for the use of gene transfer to provide information on the mechanisms of marrow reconstitution and on the source of relapse after ABMT for AML.

At present it is unknown whether contaminating malignant cells in harvested BM ever contribute to relapse of malignant disease. Several groups have used a variety of methods to purge marrow of residual malignant cells, but it is exceedingly difficult to determine the success of these efforts,1,3,6,7 Because purging is performed in the absence of measurable marrow disease, there are no quality control procedures to assess the efficacy of these procedures. Moreover, in the leukemias, the identity of the repopulating malignant cell remains unknown. Purging efficiency in preclinical models can only be assessed by the eradication

Table 1. Probability Table of Failing to Detect a Marked Relapse Secondary to Marrow-Derived Tumor (% of time no cancer cells marked)

<table>
<thead>
<tr>
<th>AML Cells Marked (%)</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>300</th>
<th>1,000</th>
<th>3,000</th>
<th>10,000</th>
<th>30,000</th>
</tr>
</thead>
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<tr>
<td>0.1</td>
<td>99.9</td>
<td>99.0</td>
<td>90.5</td>
<td>74.1</td>
<td>36.8</td>
<td>4.97</td>
<td>4.5E-3</td>
<td>9.2E-12</td>
</tr>
<tr>
<td>0.3</td>
<td>99.7</td>
<td>97.0</td>
<td>74.0</td>
<td>40.6</td>
<td>4.96</td>
<td>0.012</td>
<td>8.9E-12</td>
<td>ND</td>
</tr>
<tr>
<td>1</td>
<td>99.0</td>
<td>90.4</td>
<td>36.6</td>
<td>4.9</td>
<td>4E-3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3*</td>
<td>97.0</td>
<td>73.7</td>
<td>4.75</td>
<td>0.011</td>
<td>5.9E-12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>90.0</td>
<td>34.9</td>
<td>2.8E-3</td>
<td>1.9E-12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are percentage of time no marked cells are detected.
Abbreviation: ND, not done.
*Estimated percentage of AML cells marked (10% of cells in 30% of marrow).

Fig 4. Normal progenitor cells from patients recovering from chemotherapy can express the transferred NEO-R gene. Marrow from (■) patients recovering from chemotherapy or from (□) normal donors (see Table 1) were transduced with LNL6 and grown in methylcellulose cultures with and without G418 1.0 mg/mL. Colonies were identified and enumerated. Data show mean ± SEM for patients (n = 10) and normal controls (n = 7). (A) Numbers of colonies transduced in the two groups. (B) Transduction efficiency.
of clonogenic progenitors and there are no available methods to confirm that these cells are identical to the malignant repopulating cells. Thus, the ability of purging to reduce relapse rates remains unproven, but there is no doubt that the technique almost always damages normal marrow progenitor cells, slowing engraftment. It is therefore essential to determine whether malignant cells from the harvested marrow do contribute to relapse after ABMT and, if so, to discover whether purging can in fact remove repopulating malignant cells.

Randomized prospective clinical trials of purging versus no purging of autologous marrow may provide indirect evidence of the importance of marrow contamination. However, even if purging proved to be both necessary and reasonably effective, any individual technique is unlikely to eradicate every malignant cell in the marrow of every patient, so that differences in the relapse rates in the comparison groups would be small. Thus, substantial numbers of patients would be required to show conclusive benefit from marrow purging. This problem would be further accentuated if attempts were then made to compare the efficacy of available purging techniques.

Gene marking of marrow should complement such prospective trials and might allow evaluation of the efficacy of competing marrow-purging methodologies. Retrovirus vectors have been successfully used to mark malignant cells and cell lines in humans and in rodent models, marked malignant cells have reestablished malignant disease. Probability tables based on the present study suggest that, even with a mean of only 10% of clonogenic malignant cells marked and only a 30% aliquot of marrow exposed to the vector, there is a greater than 95% likelihood of detecting a

![Selected Colonies](790 bp —)

![Unselected Colonies](790 bp —)

**Fig 5.** Southern blot of PCR products from remission patient’s normal colonies, grown with or without G418 after transduction. Upper panel: (a) lanes 1 through 9 are colonies from selective medium; (b) lane 10 is a negative control; (c) lanes 11 through 14 are colonies from selective media; (d) lane 15 is a negative control; (e) lanes 16 through 20 are colonies from selective media; (f) lane 21 is a negative control; (g) the final three lanes are clones from selective media. The lower panel shows PCR/Southern blot of normal colonies picked from unselective media. There were only two positive colonies of a total of 20 picked.

**Fig 6.** NEO-R expression in clonogenic cells obtained from long-term marrow cultures. (A) and (B) show the numbers of G418-resistant colonies of each type obtained at weekly intervals of long-term cultures of a representative normal (A) and patient (B) marrow. (C) The change in the mean percentage of NEO-R colonies with time in nine long-term cultures (four patient, five normal). Resistant colonies are present as a stable percentage of the total for 8 to 10 weeks before beginning their decline. (■) CFU-E; (+) BFU-E; (*) GEMM; (□) G; (X) GM; (○) M.
marked relapse in any individual, provided at least 300 cells contribute to the relapse. At present, we have no way of assessing the "clonality" of relapse and hence of determining the likely validity of assumptions regarding the number of cells that contribute to relapse. However, given the relative insensitivity of current techniques for minimal residual disease detection, a marrow harvested in remission may contain upwards of 10^6 residual blasts. Fewer than 1 in 10,000 of these cells would need to contribute to a relapse for the event to be marker-positive. It should be noted that success is also dependent on two other assumptions: that the efficiency of marking leukemia cells capable of repopulating the patient is of the same order of magnitude as the frequency of in vitro marking of clonogenic leukemia cells, and that transduction does not downregulate the growth of repopulating leukemia cells.

A distinctive application of this approach is the retrovirus-mediated marking of normal progenitor cells. Gene marking studies in rodents and primates have provided information about the clonality of marrow recovery after ABMT and could also be used to study the effects of endogenous and exogenous growth factors. Such information is currently unobtainable regarding human ABMT and would be of considerable scientific and clinical value.

We studied transfer into marrow progenitors from normal donors and from patients who had received chemotherapy for AML and were candidates for ABMT. The pattern and frequency of progenitors transduced appeared identical for the two groups, with the exception of lower efficiency of transduction of the least committed (GEMM) progenitor cells. Efficiency levels are also comparable with rates of transfection previously reported on cocultivation or infection of marrow from normal donors and patients with chronic myelogenous leukemia (CML). In cultures of marrow from both groups studied here, transduced precursor cells were relatively long lived, and could be detected in vivo in clonogenic assays over an 8- to 10-week period before disappearing.

Many, though not all, animal studies would suggest that the relatively low efficiency and short persistence of marking obtained in the present preclinical studies will nonetheless be sufficient to allow marked cells to be detected in marrow and blood for weeks or months after ABMT using PCR analysis of cell subpopulations or of individual marrow progenitor colonies grown in vitro (Fig 5). Detection of these marked cells would afford an opportunity to discover whether recovery of the transplanted marrow could be modified by ex vivo or in vivo exposure to hematopoietic growth factors, and allow distinction between recovery of infused marrow and reconstitution from endogenous precursor cells resistant to supralethal chemomirradiation. In addition, if 5% or more of marrow cells are marker-positive, Southern blot analysis of proviral insertion sites would permit assessment of the clonality of marrow reconstitution. Finally, analysis of the effects of cytokine pretreatment of marrow exposed to the vector ex vivo on subsequent marker gene frequency and persistence in marrow cells in vivo will facilitate development of therapeutic gene transfer studies.

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An approach for the analysis of relapse and marrow reconstitution after autologous marrow transplantation using retrovirus-mediated gene transfer

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