Gene Transfer Into Normal Human Hematopoietic Cells Using In Vitro and In Vivo Assays

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The ability to transfer new genetic material into human hematopoietic cells provides the foundation for characterizing the organization and developmental program of human hematopoietic stem cells. It also provides a valuable model in which to test gene transfer and long-term expression in human hematopoietic cells as a prelude to human gene therapy. At the present time such studies are limited by the absence of in vivo assays for human stem cells, although recent descriptions of the engraftment of human hematopoietic cells in immune-deficient mice may provide the basis for such an assay. This study focuses on the establishment of conditions required for high efficiency retrovirus-mediated gene transfer into human hematopoietic progenitors that can be assayed in vitro in short-term colony assays and in vivo in immune-deficient mice. Here we report that a 24-hour preincubation of human bone marrow in 5637-conditioned medium, before infection, increases gene transfer efficiency into in vitro colony-forming cells by sixfold; interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) provide the same magnitude increase as 5637-conditioned medium. In contrast, incubation in recombinant growth factors IL-1, IL-3, and granulocyte-macrophage colony-stimulating factor increases gene transfer efficiency by 1.5- to 3-fold. Furthermore, preselection in high concentrations of G418 results in a population of cells significantly enriched for G418-resistant progenitors (up to 100%). These results, obtained using detailed survival curves based on colony formation in G418, have been substantiated by directly detecting the neo gene in individual colonies using the polymerase chain reaction. Using these optimized protocols, human bone marrow cells were genetically manipulated with a neo retrovirus vector and transplanted into immune-deficient bg/nu/sid mice. At 1 month and 4 months after the transplant, the hematopoietic tissues of these animals remained engrafted with genetically manipulated human cells. More importantly, G418-resistant progenitors that contained the neo gene were recovered from the bone marrow and spleen of engrafted animals after 4 months. These experiments establish the feasibility of characterizing human stem cells using the unique retrovirus integration site as a clonal marker, similar to techniques developed to elucidate the murine stem cell hierarchy.

The mature cells within the hematopoietic system have a finite life span and are continuously being replenished by the proliferation and differentiation of lineage-specific progenitor cells derived from pluripotent hematopoietic stem cells. Our knowledge of the regulation of this complex cell system derives primarily from studies in the mouse, largely because of the development of in vivo assays of the various cells within the murine stem cell hierarchy. Gene transfer has proved to be an important tool with which to characterize the murine hematopoietic system and highly infectious retroviruses are currently the most suitable vectors because stem cells are present at low frequency in the bone marrow. Conditions have been established that permit infection of a substantial proportion of murine pluripotent stem cells with retrovirus vectors containing selectable genes. The efficiency of gene transfer is governed by several factors, including virus titer. Marked improvements in gene transfer have been obtained by incubating the murine bone marrow cells in various hematopoietic growth factors, before and during the infection step. The largest increases were reported using interleukin-3 (IL-3), IL-6, or leukemia inhibitory factor (LIF), probably because these factors will stimulate the earlier progenitor and stem cells. Retroviruses require cell proliferation for integration and, because the earlier hematopoietic cells are generally out of cycle, these cells should be more infectable if they can be stimulated to proliferate. The random integration of retroviruses into murine stem cells is a powerful approach for marking individual stem cells and following their developmental potential over long periods of time during reconstitution of genetically anemic or lethally irradiated recipients. Maintaining high levels of expression from the newly introduced sequences has proved to be much more difficult because retrovirus gene expression is often downregulated in spleen colonies or long-term reconstituted mice. This downregulation may be vector-dependent because there are now several examples where vectors containing the human adenosine deaminase (ADA) gene have been expressed to high levels in long-term reconstituted animals.

Gene transfer also offers an important approach for increasing our understanding of the organization and normal developmental program of human hematopoietic stem cells. Furthermore, the correction of certain human genetic defects might be possible by introducing functional genes into repopulating stem cells. While the same gene transfer methods developed using murine cells have been successfully applied to human hematopoietic progenitor cells, the efficiency of gene transfer was lower than in the mouse. Furthermore, it is impossible to measure gene transfer into early pluripotent human stem cells because there are no assays that are equivalent to the murine short-term and long-term reconstitution assays. One approach has been to transfer genes into human cells that can be established in long-term marrow cultures (LTMC). Cells that contained

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Submitted June 1, 1990; accepted March 29, 1991.

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Supported by grants from the Medical Research Council of Canada (MRC) and the National Cancer Institute of Canada (NCIC). S.K.R. was the recipient of an MRC Post-Doctoral Fellowship and J.E.D. is a Research Scientist of the NCIC.

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and expressed the vector sequences were detected for as long as 4 to 6 weeks; this length of time should be sufficient to ensure measurement of gene transfer into earlier progenitor cells.\textsuperscript{25-28} Although the exact relationship of the long-term culture-initiating cell and a pluripotent stem cell is unknown, the available evidence suggests this cell is earlier in ontogeny than progenitors that form colonies in vitro cultures.\textsuperscript{25}

The recently developed approaches to transplant normal human hematopoietic cells into immune-deficient bgmu/ scid\textsuperscript{26-28} and scid\textsuperscript{29,30} mice may provide a foundation to develop a long sought after in vivo stem cell assay. Human myeloid progenitors have been detected in engrafted bgmu/scid mice for as long as 8 months (unpublished, January 1991); combined with the large expansion seen in the first 2 weeks after the transplant, these data provide indirect evidence that the cell type responsible for engraftment has considerable self-renewal potential.\textsuperscript{26-28} In addition to myeloid progenitors, human lymphoid cells can also be maintained in immune-deficient mice.\textsuperscript{26,30} Conclusive identification of the stem cell responsible for engraftment will require a variety of classical experimental tests that define stem cell phenotype: differentiation capacity, cycling status, self-renewal, etc.\textsuperscript{1} The ability to clonally mark individual cells with retroviruses before transplantation would clearly aid these studies.

We report the development of procedures for optimizing the efficiency of gene transfer into human hematopoietic progenitor cells. Incubation of human bone marrow in IL-6, LIF, or 5637 bladder carcinoma conditioned media (5637-CM), which contains a variety of growth factors, increases the gene transfer efficiency by at least sixfold. Because the efficiency of gene transfer using dominant selectable markers requires not only the introduction of the selectable gene but also its expression, we have analyzed single hematopoietic colonies after gene transfer for the presence of the neo gene by polymerase chain reaction (PCR). These studies gave concordance with the data generated using detailed G418-resistant (G418\textsuperscript{R}) survival curves. Finally, we provide evidence that the early cell types responsible for the engraftment of human myeloid progenitors into immune-deficient mice can be efficiently infected using retroviral vectors; progenitors that contained and expressed the neo gene were recovered from these animals for at least 4 months after transplantation.

MATERIALS AND METHODS

Gene transfer vector. The retroviral vector used in this study is the Moloney murine leukemia-derived vector, N2, whose structure has been previously described.\textsuperscript{29} Amphotropic neo virus was produced from the packaging cell line PA317-N2 at a titer of 5 x 10\textsuperscript{4} ctu/mL.\textsuperscript{29} Fresh aliquots of PA317-N2 cells were thawed every 4 to 8 weeks and no helper virus was detected in the vector-producing cell line using a sensitive marker rescue assay.

Hematopoietic progenitor cell assay. Assay for hematopoietic progenitors was performed by slight modification of an established method.\textsuperscript{30} Briefly, colonies were assayed in 0.9% methylcellulose, 30% human plasma, 5% phytohemagglutinin-stimulated leucocyte-conditioned medium (PHA-LCM), 5 x 10\textsuperscript{-4} mol/L mercaptoethanol, and 2 U/mL human urinary erythropoietin (Terry Fox Labs, Vancouver, Canada). Colony-forming unit granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) growth was enumerated with the aid of an inverted microscope (x37 magnification) after incubation at 37°C in a humidified 5% CO\textsubscript{2} atmosphere for 10 to 14 days.

Infection protocol. Human bone marrow cells were obtained from normal consenting adult donors under protocols approved by the local institutional review board, and fractionated over a Ficoll gradient. The resulting bone marrow cells were preincubated at 37°C for 24 hours in Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY) with 10% fetal calf serum (FCS; Flow Laboratories, Mississauga, Ontario) to which either 15% conditioned media (from 5637 human bladder carcinoma cells),\textsuperscript{29} recombinant human granulocyte-macrophage colony-stimulating factor (rh-GM-CSF) (1 U/mL; Genetics Institute), rh-IL-6 (10 ng/mL; Genetics Institute), or rh-LIF (50 U/mL; Genetics Institute) had been added. After 24 hours the stimulated bone marrow cells were cocultivated a further 24 hours over a subconfluent, irradiated (1,500 cGy) monolayer of PA317-N2 cells at a concentration of 1 to 2 x 10\textsuperscript{6} cells/mL. Cells were then plated in methylcellulose cultures with increasing concentrations of G418 (0 to 1,000 \mu g/mL, effective concentration). The relative colony-forming efficiency was calculated as the number of colonies scored in the presence of drug over the number in the absence of drug. Survival curves were generated by plotting the relative colony-forming ability of bone marrow CFU-GM at each G418 concentration for cells preincubated with different growth factors. The mock-infected control was bone marrow cocultivation with PA317 cells that did not produce virus. In some experiments, cells were preselected before plating by exposing them to 2 mg/mL G418 (effective concentration) for 48 hours.

PCR Single colonies were picked from methylcellulose plates, lysed at 95°C to 100°C, incubated with proteinase K (10 \mu g/mL, 2 hours at 55°C), and then prepared for PCR amplification. The buffer was composed of 67 mmol/L Tris, pH 8.8, 6.7 mmol/L MgCl\textsubscript{2}, 16.6 mmol/L (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 6.8 \mu mol/L EDTA, 10 mmol/L \beta-mercaptoethanol, 170 \mu g/mL bovine serum albumin (BSA), 1.5 mmol/L dNTP x 4, 1 pmol/L of each primer, and 2.5 U Taq polymerase in a volume of 50 \mu L. The cycling conditions were 94°C for 20 seconds, 75°C for 15 seconds, and 72°C for 15 seconds for a total of 35 cycles using a thermocycler (Bios). The oligonucleotide primers used in Figs 2 and 6B were: N1-sense, ATGCTCTCTCGATCATCCTG; and N2-sense, ATGTGCCTAGTGAATGCGGCCGTG; which flank 89 bp of the neo sequence, resulting in an amplified fragment of 141 bp in size. In Fig 3 the same primers also each contained two EcoRI restriction recognition sequences at their 5' ends and produced a 165-bp amplification product. In Fig 7 the primers were: N3-sense, TGAATGGACTCAGAGGAGGAGGC; and N5-antisense, TCTCGGCAGGAGCAAAGGAG; which flank 114 bp of the neo sequence, resulting in an amplified fragment of 141 bp in size. For personal use only.
Southern blots of some experiments were probed with the neo cDNA.

Engraftment of immune-deficient bglnuixid mice with human bone marrow. Human bone marrow, infected as described above, was infused by tail vein injection into sublethally irradiated (400 cGy) immune-deficient bglnuixid mice according to our previously reported procedures. The bglnuixid mice were bred and maintained under defined flora conditions at the Ontario Cancer Institute. As we previously reported, the health status of the animals is critically important to the success of xenografting human tissues. Animals were killed after 1 to 4 months, and the bone marrow was aspirated and plated in methylcellulose cultures as described above. Pre-tested lots of human plasma and PHA-LCM were used to selectively permit the growth of human progenitors, and in later experiments 10 U/mL rhu-IL-3 and 10 U/mL rhu-GM-CSF were used in place of PHA-LCM. Bone marrow from mice that had not been transplanted with human bone marrow served as negative controls; there were never more than 10 breakthrough colonies under these plating conditions. In some experiments, a survival curve was generated by adding 200 to 1,200 µg/mL G418 to assay for drug-resistant progenitors. Individual colonies were randomly plucked from the cultures, without G418, with a drawn out Pasteur pipette for detection of the neo gene by PCR.

RESULTS

Measurement of gene transfer efficiency by PCR. All previous studies estimating the efficiency of gene transfer into human progenitor cells have been based on in vitro colony formation in the presence or absence of drug. This biologic assay may underestimate the efficiency of gene transfer if gene expression is below the minimum level required for colony formation in the presence of the drug. Conversely, the assay could overestimate the gene transfer efficiency if functional neophosphotransferase enzyme was liberated into the media, reducing the effective G418 dose and allowing uninfected neighboring colonies to grow. In an attempt to test directly the infection efficiency, we used the PCR to detect the presence of the neo gene in individual colonies. Bone marrow was infected according to the protocol outlined and plated in the presence or absence of G418; a typical survival curve is shown in Fig 1. We routinely estimate gene transfer efficiency using detailed survival curves rather than single selective drug concentrations because the shape of the survival curve allows an accurate estimate of the size of the drug-resistant population. Furthermore, in our experience there is often a considerable degree of experimental variation using bone marrow from different donors, different cell culture conditions, and different lots of G418. Inspection of the curve generated by plating infected bone marrow in increasing concentrations of G418 shows a biphasic curve indicative of two populations of progenitors. One population of drug-sensitive progenitors is killed at the same rate as the mock-infected cells, and the other population is drug resistant. The size of the drug-resistant population can be estimated from the intersection of the slope of the line that describes this population and the vertical axis; in this case, it was about 15%. To verify these results, colonies were picked from the cultures without G418 established 10 to 14 days earlier with infected bone marrow and analyzed by PCR using neo-specific primers (Fig 2). A representative experiment testing 20 randomly picked colonies indicated that the 141-bp neo-specific fragment was amplified in the positive control samples and from four of 20 colonies or 20% infection efficiency (lanes 2, 7, 14, and 20). This value is a minimum estimate because there are very faint bands present in several other lanes and these may be small colonies that did not amplify to the same extent. The maximum estimate was seven of 20 or 35%. The controls included uninfected human bone marrow colonies, the extraction buffer that the colonies were suspended in before PCR, and methylcellulose aspirated from the same plate that the infected cells were plated in; all were negative. Similar gene transfer efficiencies (20% to 30%) into human bone marrow have been determined by PCR on over 100 colonies from six independent experiments using different vectors with similar titers that contained either the neo gene, the human adenosine deaminase cDNA, or various oncogenes (unpublished, January 1991). In all these experiments, the gene transfer efficiency as estimated from the bioassay correlated with the PCR determination.

Preselection of bone marrow cells after infection enriches for hematopoietic progenitor cells that contain and express the neo gene. Figure 1 also shows the survival curve of bone marrow that was infected and then subjected to preselection in 2 mg/mL G418 for 48 hours before plating. In contrast to the biphasic curve generated by infected but not preselected cells, the straight line indicates that preselection results in a uniform population of drug-resistant progenitors and the intersection of this line with
GENE TRANSFER INTO HUMAN HEMATOPOIETIC CELLS

Fig 2. PCR analysis of individual CFU-GM progenitor colonies from human bone marrow infected with a neo vector. The numbered lanes represent 20 CFU-GM picked at random from a methylcellulose culture without G418 that had been plated with bone marrow following preincubation and cocultivation with virus-producing fibroblasts. Following PCR for the presence of the neo gene, the reaction products were run on an agarose gel and stained with ethidium bromide. The arrow indicates the 141-bp amplified neo band using primers N1-N2. The positive controls are 500 ng of DNA from the virus producing cell line and a single hematopoietic colony from a human leukemic cell line that carries the neo gene. The variation in the intensity of the signal from the positive colonies is likely due to the fact that the colonies are of different sizes. The negative controls are uninfected human CFU-GM, the extraction buffer, and 5 μL of methylcellulose from a plate with infected CFU-GM.

the y-axis at the origin suggests the entire population is drug-resistant. To confirm this conclusion, colonies were randomly picked from infected, preselected bone marrow grown in the absence of G418 and analyzed for the presence of the neo gene using PCR. All of the colonies tested (12 of 12; only four shown, Fig 3, lanes 1 through 4) contained the amplified neo-specific band, confirming our conclusion from Fig 1 that following preselection the entire population contained the neo gene. To determine if G418 is absolutely selective for infected colonies, non-preselected colonies growing in the presence of G418 were also analyzed by PCR. As shown in Fig 3 (lanes 5 through 11) all of the colonies (7 of 7) contained the neo gene and no uninfected colonies were detected on these plates. Lane 12 is the negative control of uninfected human cells. The lower portion of the figure shows the Southern blot of the upper ethidium bromide-stained agarose gel; the positive hybridizing band confirms specific amplification of the neo gene.

Effect of growth factors on the efficiency of gene transfer. We previously showed that the addition of conditioned
media from 5637 cells to bone marrow during the 24-hour cocultivation period increased the recovery of infected progenitors by threefold. However, no evidence was obtained that the efficiency of gene transfer was increased. The experimental protocol was altered to allow a longer preincubation of bone marrow in growth factors before and during infection to permit more time for the cells to respond. In addition, a number of other recombinant growth factors (IL-1, IL-3, GM-CSF), as well as 5637-CM, were compared with each other for their ability to increase the efficiency of gene transfer into bone marrow cells from the same donor. Following preincubation with growth factors and cocultivation with virus producing fibroblasts, bone marrow cells were plated in the presence of increasing concentrations of G418. Survival curves from a representative experiment are shown in Fig 4; despite the consistent variation in the 800 μg/mL G418 point, the size of the drug-resistant population can easily be determined using the techniques described earlier. The gene transfer efficiency without prestimulation with any factor was 5%, while the addition of 5637-CM increased the gene transfer efficiency to 20%. The other factors increased gene transfer efficiency by no more than twofold if at all. The data from this experiment and four other independent experiments are summarized in Table 1. The results indicate that preincubation in 15% 5637-CM increased gene transfer efficiency by almost sixfold. In contrast, preincubation in 1 U/mL of IL-3, IL-1, or GM-CSF increased the gene transfer efficiency from 1.5- to 3-fold. Higher (10 to 50 U) and lower (0.1 U) concentrations of factors alone or in combination did not produce any further increase in gene transfer efficiency (data not shown); the overall yield of progenitor colonies was often reduced at higher growth factor concentrations.

Because IL-6 and LIF have recently been shown to increase gene transfer into murine hematopoietic cells and because 5637-CM contains these and other factors, we compared them with 5637 in the human system. The survival curves shown in Fig 5, representative of two experiments, indicate that 50 U/mL LIF and 10 ng/mL IL-6 increased the gene transfer efficiency into human progenitors by the same extent (fivelfold) as 5637-CM. Double the concentration of each factor produced the same results (data not shown).

Gene transfer into human hematopoietic cells capable of engrafting immune-deficient mice. The lack of an in vivo experimental assay for human stem cells more primitive in their ontogeny than colony-forming cells prompted several groups, including our own, to establish an animal model that may be more suitable than in vitro cultures for studying human hematopoiesis. Although it remains to be conclusively proven, preliminary evidence suggests that the cell type responsible for engrafting immune-deficient mice following transplantation with normal human bone marrow is earlier in ontogeny than the CFU-GM progenitor and perhaps earlier than all colony-forming cells. Our studies found that there was a greater than 40-fold increase in the number of CFU-GM in the first 14 days posttransplant and that this level of engraftment was maintained in animals analyzed at 5 weeks, 12 weeks, and 32 weeks (unpublished, January 1991). Because CFU-GM have very little self-renewal capacity, the large initial increase in progenitors and their maintenance for several months must be due to engraftment of an earlier cell type with extensive self-renewal capacity.
capacity for self-renewal, features characteristic of stem cells.

Retrovirus-mediated gene transfer has proved to be a powerful approach to characterize murine stem cells. In an attempt to take a similar approach for human stem cells, we have infected cells with a retrovirus vector, using the optimized procedures described above, before transplantation into immune-deficient bglnuixid mice. Normal bone marrow cells were prestimulated in 5637-CM, infected, preselected, and infused by intravenous (IV) injection into sublethally irradiated bglnuixid mice. Previous experiments on the kinetics of engraftment with normal human bone marrow showed that steady state levels of progenitors were achieved 4 weeks posttransplant; therefore, animals were killed at 5 weeks and a portion of the bone marrow was plated under conditions that only permitted human progenitors to grow. In addition, DNA was extracted from the bone marrow and spleen and analyzed by PCR for the presence of the neo gene. The expected 141-bp PCR product was seen in the positive control and in the bone

Table 1. Effect of Hematopoietic Growth Factor Addition on Retrovirus-Mediated Gene Transfer Efficiency

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. Growth Factor</th>
<th>5637-CM</th>
<th>% Gene Transfer Fold Increase</th>
<th>IL-3</th>
<th>% Gene Transfer Fold Increase</th>
<th>IL-1</th>
<th>% Gene Transfer Fold Increase</th>
<th>GM-CSF</th>
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<td>11</td>
<td>5.5</td>
<td>10</td>
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<td>19</td>
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<td>2</td>
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<td>1.7</td>
</tr>
<tr>
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Average (mean ± SD) 5.6 ± 2.7 2.7 ± 1.5 1.5 ± 0.4 3.4 ± 4

Human bone marrow cells were incubated for 24 hours before cocultivation over PA317-N2 fibroblasts in the hematopoietic growth factors indicated. The cells were then plated in increasing concentrations of G418 to generate survival curves and the efficiency of gene transfer was estimated from the survival curve. The efficiency of gene transfer was compared between cells that were not exposed to exogenous factor and those incubated in the factor indicated. The mean ± SD was calculated for the fold increase in gene transfer efficiency from five experiments and was significant (Student's t-test, P > .05) except for GM-CSF, which was not significant. The data in experiment 5 is from a single selective drug concentration rather than a survival curve.

Abbreviation: ND, not done.
marrow and spleen from both engrafted animals, indicating that the animals were engrafted with human bone marrow cells that had been infected with the neo vector (Fig 6B). To quantitate the proportion of neo-marked cells in these tissues, a multiplex PCR reaction was developed that contained neo primers as well as primers that will amplify a region of the dystrophin gene (dys) that is conserved in mouse and humans as an internal control for each reaction. Comparison of the intensity of the neo and dys amplified products to standard dilutions indicates that approximately 1% of the bone marrow and spleen contained neo vector-infected cells (Fig 6A). A total of $6.4 \times 10^4$ and $5.8 \times 10^4$ human CFU-GM was detected in the bone marrow from animals 19HM1 and 19HM2, respectively. No colonies grew in plates with increasing concentrations of G418. Colonies growing in the absence of G418 were analyzed by PCR for the presence of the neo gene and seven of 10 colonies from 19HM1 and 19HM2 contained an easily visible band while the other three colonies produced fainter bands (Fig 6B). This high percentage of infected progenitors recovered from the animals (at least 70%) was likely due to the fact the cells had been preselected before engraftment. We have observed similar high efficiency gene transfer into mice reconstituted with infected and preselected murine CFU-S and stem cells. Guided by this experience with the murine system, we tested several different regimes of factor addition as well as several different conditioned media and recombinant growth factors for their ability to increase gene transfer efficiency into human progenitors. Detailed survival curves, rather than single drug concentrations, were generated to detect small differences in the efficiency of gene transfer. Conditioned media from 5637 cells consistently produced fivefold to eightfold increases in gene transfer efficiency, if cells were preincubated in the conditioned media for 24 hours before infection. In contrast, preincubation in recombinant

**DISCUSSION**

Gene transfer is an important tool to investigate the organization and genetic mechanisms that govern the developmental program of human stem cells. Although murine studies have confirmed that retrovirus vectors are the most efficient means of introducing genes into pluripotent repopulating stem cells, it has been difficult to achieve a similarly high efficiency of gene transfer into human progenitor cells. Initial studies using protocols similar to those in the mouse indicated that the frequency of gene transfer into human cells was 1% to 5%. Studies in the mouse suggested at least three factors that provide populations of progenitor and stem cells greatly enriched for cells expressing the neo gene: pretreatment of donor mice with agents such as 5-fluorouracil (5-FU), the inclusion of growth factors during infection, and preselection of the infected cells. The first two parameters are based on the principle that retroviruses require cells to proliferate in order to integrate into the host genome and the fact that the more primitive hematopoietic cells cycle very slowly. Treatment of donor bone marrow with 5-FU enriches for non-cycling stem cells and induces these cells to cycle, while various growth factors can directly affect the proliferation of progenitor and stem cells. Experiments in the mouse found increased gene transfer efficiencies into CFU-spleen (CFU-S) and stem cells if bone marrow cells were incubated in IL-3, IL-6, LIF, or combinations before or during cocultivation. While preselection does not affect gene transfer efficiency, several studies showed significant enrichment for infected CFU-S and reconstituting stem cells following in vitro exposure to 2 mg/mL G418. Because in vivo repopulation is characterized by competition between different stem cell clones, preselection ensures animals are transplanted only with infected stem cells.

Guided by this experience with the murine system, we tested several different regimes of factor addition as well as several different conditioned media and recombinant growth factors for their ability to increase gene transfer efficiency into human progenitors. Detailed survival curves, rather than single drug concentrations, were generated to detect small differences in the efficiency of gene transfer. Conditioned media from 5637 cells consistently produced fivefold to eightfold increases in gene transfer efficiency, if cells were preincubated in the conditioned media for 24 hours before infection. In contrast, preincubation in recombinant
IL-1α, IL-3, and GM-CSF increased the efficiency by only 1.5- to 3-fold. Preliminary experiments using various combinations of these factors did not increase gene transfer efficiency any further. This finding suggests that a combination of factors in 5637-CM (IL-1, G-CSF, IL-6, LIF) or other novel factors are optimal for increasing gene transfer. This conditioned medium was also found to be effective in our murine stem cell infection protocols. The component(s) of 5637-CM responsible for this increase in gene transfer efficiency were IL-6 and LIF because they both provided the same magnitude increase as 15% 5637 medium. Incubation of human bone marrow cells during cocultivation in agar-LCM plus GM-CSF/IL-1β or a stromal cell-conditioned medium can also increase gene transfer efficiency by threefold to sevenfold.

Most gene transfer experiments using drug-resistance genes have been based on colony formation in drug and, therefore, require gene expression to detect infection. There are several reasons why it would be important to develop an assay independent of growth in drug. Many genes that are important in cell biologic experiments or that can be used for gene therapy applications are not selectable, requiring the construction of complex vectors containing multiple genetic elements. A number of murine studies have pointed to the fact that retrovirus vector gene expression is sometimes downregulated in hematopoietic cells. If this downregulation occurs in colony forming cells, then the infection frequency will be underestimated. Furthermore, we have found wide variations in the cytotoxicity of different batches of G418, sometimes making it difficult to reproducibly obtain accurate estimates of gene transfer efficiency. The advent of PCR is a solution to each of these problems. To directly test the gene transfer efficiency, we used the PCR technique to detect the newly introduced neo gene.
gene in individual colonies. This direct assay correlated well with the efficiency estimated from survival curves of colony formation in drug. If only single drug concentrations are used, PCR analysis provides a higher estimate of the actual gene transfer efficiency than predicted by the bioassay because the drug selection conditions also kill infected progenitors at increased drug concentrations (ie, the slope of the line describing the resistant population is usually less than 0). In addition, we have previously reported that preselection of infected human bone marrow yields a population where all of the progenitor cells contain the neo gene.\textsuperscript{22} PCR analysis again confirmed that all the colonies that arose from infected and preselected bone marrow contained the neo gene. Finally, the PCR analysis indicated that no breakthrough of uninfected colonies occurred at high doses of G418 because all colonies obtained from infected bone marrow growing in G418 contained the provirus.

The long-term goals of these studies are to genetically manipulate human stem cells with retrovirus markers to characterize the cells that comprise the human stem cell hierarchy, to introduce growth regulatory genes into stem cells to perturb their developmental program, and to measure the long-term expression of clinically relevant genes in human stem cells as a prelude to gene therapy. A major problem has been the lack of a suitable assay for primitive pluripotential human stem cells. The human LTMC system, if followed for longer than 4 to 6 weeks, is
able to detect cells that may be earlier in ontogeny than in vitro colony-forming cells. In an elegant series of experiments, Sutherland et al have modified the LTMC system and measured the frequency of the initiating cell in unseparated and highly purified human bone marrow by limiting dilution. Hughes et al have recently reported transfer of the neo gene into human bone marrow cells before establishment in LTMC; significant levels of drug-resistant progenitors were recovered for up to 6 weeks. Using a similar infection protocol, we have confirmed these results, and using a sensitive quantitative Southern blotting technique, showed that a significant proportion of the nonadherent and adherent cells contain the gene at 6 weeks. Human ADA gene expression was also detected in LTMC established from ADA vector-infected bone marrow, although the cultures were only followed for 3 weeks. With further characterization, the human LTMC system will be an important in vitro method to study human hematopoietic cells; however, it is currently severely limited because cells can only be maintained in LTMC for rather short periods of time before the output of progenitors drops markedly and current culture conditions favor granulocyte/macrophage lineages and do not support lymphoid cells.

The recent description of methods to transplant normal and leukemic human hematopoietic cells into immune-deficient mice lays the foundation for an alternate approach to study human progenitor cells and stem cells. We have found that a variety of human hematopoietic cells can be engrafted into immune-deficient mice, including normal human bone marrow, various leukemic cell lines, and leukemic bone marrow taken directly from patients (Kamel-Reid et al, submitted). It is also possible to engraft human lymphoid cells into immune-deficient mice.

Retrovirus tagging of bone marrow cells before engraftment in immune-deficient mice offers an important approach to characterize the cell types responsible for maintaining the engrafted human progenitors that can be detected in the mice for long periods of time. Furthermore, gene transfer of clinically relevant genes into these mice will provide an important system for future gene therapy trials in humans. The experiments reported here show that a large proportion (20% to 70%) of the human progenitors recovered from engrafted animals contained the neo gene for up to 4 months posttransplant. While we have not yet attempted to engraft animals with bone marrow that was not preselected, this high recovery of infected progenitors may be due to the preselection step. Drug-resistant progenitors were detected in one experiment, indicating that the neo gene was expressed in a proportion of the progenitor cells. Our inability to detect any G418 progenitors in earlier experiments was probably due to the batch of G418 used and the fact that the progenitor colonies derived from the engrafted mice are often small. Conclusive evidence that a stem cell has engrafted these mice will require demonstration of a common retrovirus integration site in hematopoietic cells of different lineages. Our long-term goal, not yet realized, is to establish both lymphopoiesis and myelopoiesis in immune-deficient mice transplanted with human bone marrow, thereby providing the opportunity to investigate pluripotent stem cells. In the case where only single lineages are present, it should still be possible to use PCR strategies to clone out flanking sequences from the individual infected myeloid progenitor colonies to determine if they came from a common infected precursor.

Taken together, high efficiency gene transfer procedures and novel in vivo assays for human hematopoietic cells will allow the manipulation of human hematopoietic stem cells in ways that were previously only possible in the mouse.

ACKNOWLEDGMENT

We thank S. Clark for hematopoietic growth factors and R.A. Phillips for critically reviewing the manuscript.

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Gene transfer into normal human hematopoietic cells using in vitro and in vivo assays

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