Autologous Transplantation of Canine Long-Term Marrow Culture Cells Genetically Marked by Retroviral Vectors


Retroviral infection of bone marrow cells in long-term marrow cultures (LTMCs) offers several theoretical advantages over other methods for gene transfer into hematopoietic stem cells. To investigate the feasibility of this approach in a large animal model system, we subjected LTMCs from nine dogs to multiple infections with retrovirus containing the neomycin phosphotransferase gene (neo) during 21 days of culture. Feeder layers, cocultivation, polycations, and selection were not used. The in vitro gene transfer efficiency was 70% as determined by polymerase chain reaction amplification of neo sequences in colony-forming unit granulocyte-macrophage (CFU-GM) obtained from day-21 LTMCs. Day-21 LTMC cells were infused into autologous recipients with (four dogs) and without (three dogs) marrow-ablative conditioning. At 3 months posttransplant, up to 10% of marrow cells contained the neo gene. This percentage declined to 0.1% to 1% at 10 to 21 months posttransplant. Neo was also detected in individual CFU-GM, burst-forming unit-erythroid (BFU-E), and CFU-Mix progenitors derived from marrow up to 21 months postinfusion and in cultures of peripheral blood-derived T cells up to 19 months postinfusion. There was no difference in the percentage of neo-marked cells present when dogs that received marrow ablative conditioning were compared with dogs receiving no conditioning. Detection of neo-marked marrow cells almost 2 years after autologous transplantation in a large mammalian species shows that retroviral infection of marrow cells in LTMCs is a potentially nontoxic and efficient protocol for gene transfer. Further, our results suggest that marrow conditioning and in vivo selection pressure to retain transplanted cells may not be absolute requirements for the retention of genetically marked cells in vivo.

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Gene Transfer technology provides a powerful approach to the modification and study of cellular function. Specific applications include the treatment of genetic disease, cancer therapy, and characterization of the developmental program of hematopoietic cells. The target in most of these applications is the pluripotent stem cell present in the bone marrow (BM), but the low frequency of stem cells necessitates a highly efficient gene transfer system. To date, retroviruses have been the most useful vectors for gene transfer. Foreign genes have been introduced in vitro into hematopoietic progenitors of many species including the mouse, dog, nonhuman primates, and human. Gene transfer and expression into pluripotent stem cells capable of long-term reconstitution has only been shown for the mouse, using a variety of vectors. Despite these successes, major issues remain to be resolved before therapeutic measures using gene transfer into human BM can be considered feasible.

One of these issues is the need to develop methods to transfer genes of interest at high efficiency into stem cells capable of reconstituting the hematopoietic system of large animals. In general, marrow cells infected in vitro have not been maintained at high levels in vivo after autologous transplantation. This result has been attributed to the inability to infect a sufficiently large number of primitive stem cells with reconstituting capacity. Most infection protocols involve short-term (1 to 3 days) cocultivation of BM cells with virus-containing media, or cocultivation over irradiated monolayers of virus-producing fibroblasts (reviewed by Kohn et al'). Evidence indicates that cell cycling may be necessary for retroviral reverse transcription and integration,' and this suggests that a possible reason for the failure of retroviral vectors to transduce foreign DNA into primate BM stem cells may be that they are noncycling in unmanipulated marrow. Further, there appear to be properties intrinsic to adult as opposed to fetal marrow cells that may contribute to the failure of retroviral vectors to infect unmanipulated adult marrow stem cells.

Experiments in the mouse have clearly shown that incubation of BM with hematopoietic growth factors before infection increases gene transfer efficiency, presumably by stimulating the stem cells into cycle. Similar approaches using human progenitors have resulted in a fivefold to 10-fold increase in gene transfer efficiency. One of the major problems with these short-term in vitro infection schema is the loss of stem cells. This is especially true for procedures that involve cocultivation over irradiated virus-producing fibroblasts in which stem cells, which have adherent properties, may be lost on the fibroblasts.

Retroviral infection combined with Dexter-type long-term marrow culture (LTMC) offers opportunities for improved gene transfer efficiency through manipulation of...
adult marrow progenitor cells.\textsuperscript{20,22,26} We describe here a strategy for gene transfer into LTMC-derived canine hematopoietic progenitors in which cells in LTMCs were subjected to repeated retroviral infections timed to coincide with the wave of cellular proliferation thought to occur after media replenishment.\textsuperscript{1,13,15} We reasoned that with this protocol, primitive progenitor cells could be maintained in the adherent layer and repeatedly triggered into cycle, thus providing the opportunity for optimizing retroviral infection over a long period of time. We used a canine model system, as this is an animal model intermediate in size to mice and humans and one with which we have experience.\textsuperscript{26}

We subjected LTMCs from normal dogs to three rounds of infection with the neo-containing retrovirus produced by the packaging cell line PA317-N2\textsuperscript{24,37} during 21 days of culture. Our results showed that the neomycin phosphotransferase gene (neo) was transferred with high efficiency into hematopoietic progenitors in LTMCs. After infusion of infected LTMC cells into autologous recipients, we detected progeny cells of genetically marked marrow progenitors, with and without the use of marrow conditioning by total body irradiation (TBI). This latter observation supports the view that marrow ablative conditioning may not be required for engraftment of LTMC-derived progenitors. Results from this large animal model indicate that our method, which does not depend on selection, growth factors, polyclonies, cocultivation, or feeder layers, is a nontoxic approach for genetically marking marrow progenitor cells with at least some reconstituting potential.

**MATERIALS AND METHODS**

Normal dogs were obtained from the Central Animal Facility of the University of Guelph and maintained in the Veterinary Teaching Hospital until they recovered from all procedures. All protocols were approved by committees of the University of Toronto, The Toronto Hospital, and the University of Guelph concerned with animal care, biohazards, and ethical review.

**Preparation of viral supernatants.** The neo retroviral vector used was the Moloney murine leukemia-derived vector, N2.\textsuperscript{24,37} Before marrow harvest, the helper cell line PA317-N2 was grown in α-MEM media containing 4.5 g/L L-glutamine, 10% fetal bovine serum, and 0.2 g/L G418. The day before marrow harvest, the α-MEM media was replaced by media used for LTMCs.\textsuperscript{37} On the day of the marrow harvest, this media was collected and a cell-free supernatant containing ~1 × 10^8 virus/mL in long-term culture media was prepared by centrifugation at 400g for 15 minutes. Aliquots of virus-containing media were similarly prepared for second and third infections of each sample. No helper virus was detected by a sensitive viral rescue assay in these viral supernatants or from the LTMCs after 21 days.

**Gene transfer into canine LTMC.** After induction of general anesthesia, marrow and blood cells were aspirated from the iliac crests, ischiatric crests, and proximal femori and humeri of each dog, and placed directly into preservative-free heparin (2,000 U/100 mL of aspirated marrow). After determination of the viable nucleated cell count by trypan blue exclusion, the mononuclear cell fraction was prepared by density centrifugation and LTMCs were initiated as previously described\textsuperscript{\textsuperscript{15}} along with the following modifications: each marrow culture was set up in culture medium containing retrovirus and exposed to virus on two more occasions during the culture period, with the last exposure to virus occurring 6 days before culture harvest and infusion at 21 days. Specifically, cultures were initiated by inoculating Corning T-150 flasks (Corning, Corning, NY) with 1.2 × 10^8 mononuclear cells in 60 mL of media containing virus. On days 7 and 14 of incubation, the flasks were semi-depopulated and fed with non–virus-containing media. On days 8 and 15, one-half of the supernatant layer of each flask was removed, the cells were pelleted and resuspended in fresh media containing ~1 × 10^8 virus/mL. After 21 days of culture, all adherent cells were recovered by trypsinization, washed three times in preservative-free, pH indicator-free Hank's buffered saline solution (GIBCO, Grand Island, NY), and kept at room temperature for 1 to 3 hours before bolus injection by syringe and butterfly needle infusion set into the cephalic vein of the autologous recipient.

**Progenitor assays.** Progenitor cells (colony-forming unit granulocyte-macrophage [CFU-GM], burst-forming unit-erythroid [BFU-E], CFU-Mix) were assayed, at least in duplicate, in 1 mL of methylcellulose media plated in 35 mm petri dishes as previously described.\textsuperscript{\textsuperscript{37}} Resistance to the neomycin analog, G418, was assessed by counting the number of colonies arising by day 10 in plates containing 1 mg/mL G418 compared with plates containing no added G418. For evaluation of T cells, nucleated peripheral blood cells were maintained in liquid culture consisting of RPMI 1640 (GIBCO), 10% fetal calf serum (FCS), 1% L-glutamine, and 1% pen/strep, at 10^6 cells/mL. Phytohemagglutinin (PHA; Wellcome, Dartford, UK) was added for 72 hours at culture initiation after which time cells were pelleted by centrifugation and resuspended in fresh media supplemented with interleukin-2 (Cetus, Norwalk, CT) at 20 U/mL twice weekly for 2 to 3 weeks.

**Detection of neo by polymerase chain reaction (PCR).** Approximately 10^6 cells from fresh blood, BM, cultured T cells, or LTMCs were lysed and RNA was extracted for PCR amplification, DNA from day-10 CFU-GM, growing in the absence of G418 and estimated to contain at least 20 cells, was obtained for PCR amplification by plating diluted colonies directly into 19 mL of extraction buffer (50 mmol/L KCl, 10 mmol/L Tris HCl pH 8.3, 2.5 mmol/L MgCl₂, 0.01 mg/mL 2% gelatin, 0.45% nonidet P40, 0.45% Tween 20) and incubating with 10 μg proteinase K for 2 hours at 55°C. The neo-specific oligonucleotide primers were a 21mer to the sense strand (5′ AAC GA TT C AT C AT TG G CT G T A 3′) and a 20mer to the antisense strand (5′ ATGC TCT CTG TC CC AG AT CAT 3′). Neo-specific sequences were amplified according to standard Cetus protocol\textsuperscript{26} from 250 ng to 1 μg extracted DNA or DNA obtained by direct lysis of plated colonies. The denaturation temperature was 94°C, the annealing temperature was 68°C, and the extension temperature was 72°C. The amplified DNA was identified after electrophoresis in 1% agarose and 2% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME) by staining with ethidium bromide. Southern blots were prepared by transferring the DNA from the gel to a nylon membrane (Hybond; Amersham, Oakville, Ontario, Canada). Blots were probed with a 32P-labeled neo fragment. Specificity was determined by autoradiography with Kodak X-omat film (Rochester, NY). Control specimens included serial dilutions of PA317.N2 and/or HL-60.N2 (an HL-60 cell line containing a neo vector), reagents only, BM cells from uninfected normal dogs, and CFU-GM derived from assays of uninfected normal dogs, and CFU-GM obtained from marrow conditioning and support protocols have been described elsewhere.\textsuperscript{1,13,15} Marrow conditioning was administered to transplanted dogs and control dogs by bilateral exposure to a single 60Co source; the total midline dose was 6 Gy or 8 Gy in a single fraction at dose
rates from 10 cGy/min to 75 cGy/min. Dogs receiving transplants without marrow conditioning were not irradiated and were not administered antibiotics. Followup specimens of peripheral blood and BM (obtained under general anesthesia) were obtained at intervals and assayed as described above by PCR amplification for the presence of neo.

Detection of infectious neo virus and replication competent retrovirus in the plasma of reconstituted dogs. The PA317-N2 cell line was re-established from frozen stocks every 6 weeks to prevent the inadvertent production of replication-competent virus. Plasma from reconstituted dogs was tested for the presence of infectious neo virus using Rat 2 cells as an indicator line. Dog plasma was diluted to 10% with α-MEM containing 8 μg/mL polybrene and incubated for 24 hours with 2 × 10⁶ Rat 2 cells on 60 mm tissue culture plates. Higher plasma concentrations caused Rat 2 cells to grow poorly. After 24 hours, fresh α-MEM with 400 μg/mL G418 was added to the plates. The plates were incubated for 7 days and scored for G418⁺ colonies. The positive control was the PA317-N2 cell line.

The dog plasma was also tested for the presence of replication-competent helper virus using a marker rescue assay. The diluted plasma, α-MEM, and polybrene were incubated with NeoNP-1 cells for 24 hours. NeoNP-1 is a 3T3 cell line infected with the MLVneo.l retrovirus vector. Any helper virus in the test sample will spread throughout the culture and rescue the MLVneo.l vector that can then be assayed on Rat 2 cells or human HOC-7 cells. The NeoNP-1 cells were passaged and grown for at least 1 week to permit virus spread. This supernatant was diluted from 10⁶ to 10⁶³ and incubated with Rat 2 cells to assay for the rescued MLVneo.l vector. The positive control was supernatant from a packaging cell line known to make helper virus and the negative control was supernatant from the NeoNP-1 cell line.

RESULTS

Tables 1 and 2 summarize the procedures and results for nine dogs. Four dogs received the entire transplant protocol including marrow conditioning ("MC⁺", dogs 1 through 4). The first dog died due to a failure of support (fulminant Streptococcal septicemia) at 18 days postirradiation, but dogs 2, 3, and 4 survived and were evaluated for evidence of retention of genetically marked hematopoietic cells. Three dogs received the entire protocol except that marrow conditioning was completely omitted ("MC⁻", dogs 5, 6, and 7). Dogs 8 and 9 were controls that received the entire protocol except that no cells of any kind were infused after marrow conditioning; both recovered from pancytopenia at approximately 35 days but dog 9 died after a severe acute reaction to a platelet transfusion at 36 days.

Marrow harvests and cell recovery from cultures. Figure 1 summarizes the numbers of cells harvested and then obtained after 21 days of LTMC. The mean number of mononuclear cells initially harvested was 1.6 × 10⁸ (range, 3.1 × 10⁷ to 2.3 × 10⁸) per kilogram of body weight (BW), which corresponded to a mean of 1.2 × 10⁸ (range, 4.6 × 10⁷ to 2.5 × 10⁸) CFU-GM/kg BW based on colony assays. Pelvic aspirates provided the highest density of mononuclear cells. Good harvests were difficult to obtain from dogs weighing ~10 kg or less because the pelvis tended to be too small to allow extensive aspiration; the aspirated marrow was often highly diluted with blood, as indicated by the small proportion of mononuclear cells obtained after density gradient centrifugation. Harvest
Table 2. Summary of Results: In Vitro Gene Transfer Efficiency

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<th>Dog</th>
<th>1</th>
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<th>6</th>
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<th>8</th>
<th>9</th>
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<th>SD</th>
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<td>10</td>
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<td>28</td>
<td>61</td>
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<td>24.5</td>
</tr>
<tr>
<td>% Positive by PCR</td>
<td>100</td>
<td>33</td>
<td>71</td>
<td>73</td>
<td>66</td>
<td>61</td>
<td>71</td>
<td>87</td>
<td>74</td>
<td>70.7</td>
<td>18.3</td>
</tr>
</tbody>
</table>

Data based on CFU-GM derived from day-21 LTMCs. Adherent layer cells were harvested by trypsinization and assayed (at least in duplicate) in methylcellulose suspension. For % G418 resistance, a minimum of 50 CFU-GM was assayed with and without 1 mg/mL G418 and counted at 10 days. For % positive by PCR, 10 to 25 day-10 non-G418-resistant CFU-GM were individually plucked and analyzed by PCR for each dog.

Volumes ranged from 110 mL to 300 mL, representing 10% to 20% of blood volumes, and transfusions were not required. After culture, the mean number of cells recovered was $1.9 \times 10^7$ (range, $2.7 \times 10^6$ to $4.5 \times 10^7$/kg BW, corresponding to a mean of $8.5 \times 10^2$ (range, $2.0 \times 10^2$ to $2.9 \times 10^3$) CFU-GM/kg BW. Thus, on average, the number of cells recovered at the end of culture was 12% of the initial number of mononuclear cells seeded, while a smaller proportion of the initial number of CFU-GM, about 7%, was recovered after culture.

Efficiency of gene transfer in vitro. Table 2 summarizes data obtained from assays of G418 resistance, and from detection of the neo gene in non-G418-resistant CFU-GM that were individually plucked (10 to 25 for each dog) and subjected to PCR amplification. In some cases, such as in dog 3, there was a large difference between neo expression, as determined by G418 resistance, and the presence of neo-amplifiable sequences, as determined by the percentage of PCR-positive colonies. The proportion of CFU-GM resistant to G418 exhibited wide variation about a mean of 39% and was always less than the efficiency of gene transfer measured by PCR. A titration curve for G418 resistance is shown in Fig 2A. We found wide variation in nonspecific toxicity with different lots of G418. Because this was a bioassay, any factors that inhibited colony growth would have led to underestimates of gene expression. The mean gene transfer efficiency as measured by PCR was approximately 70%, and ranged from 30% in dog 3 to 100% in dog 1. The extremely high rate of gene transfer achieved for cultures from dog 1 was evident when all CFU-GM colonies plucked for PCR amplification were positive for neo (Fig 2B).

Studies of gene transfer efficiency in cultures after each exposure to infection showed that there was a weak increase in the proportion of genetically marked cells. This increase was especially evident after the final infection (Fig 3). Individually plucked CFU-GM exhibited a similar increase in gene transfer efficiency and G418 resistance (data not shown).

Transplantation of genetically marked LTMC cells into autologous recipients. By microscopic examination, the trypsinized cell suspension obtained for transplantation consisted largely of single mononuclear and spindle-shaped cells along with occasional multicellular clumps of more than 100 cells. Adverse reactions to bolus intravenous infusion of the cell suspensions were not noted. MC+ and control dogs underwent a period of prolonged pancytopenia marked by febrile episodes, leading to recovery of cells (neutrophils >1.0 x 10^9/L) by about 36 days and platelets (>50 x 10^9/L) by about 42 days postirradiation. MC− dogs did not develop any physical or hematologic abnormalities. The procedures performed, dosages of cells injected, and duration of follow-up for each dog are outlined in Table 1.

Specimens of peripheral blood obtained from MC+ and MC− dogs at 2 weeks to 21 months after infusion of cultured cells were consistently positive for neo, as shown by PCR amplification (Fig 4). Similarly, specimens of BM obtained from MC+ dogs (from 1 month to 21 months after infusion of cultured cells) and MC− dogs (from 1 month to 19 months after infusion) were consistently positive for neo by PCR amplification (Table 3 and Fig 5). All five dogs tested were also positive for the neo marker when peripheral blood activated T cells were assayed (Table 3). Semi-quantitative estimates of the retention of neo were made by comparing standardized dilutions of the neo amplification product, visualized in gels and Southern blots, with control samples of 100%, 10%, 1%, and 0.1% dilutions of the cell lines HL60, N2, or PA317. N2 with the respective parent cell lines (HL60 and PA317) after similar amplifications (Fig 5 and Table 3). In all dogs that received infected cells, the proportion of neo-marked cells declined over the first 3 to 4 months postinfusion of LTMC cells. The three MC+ dogs with follow-up at 21, 17, and 14 months (dogs 2, 3, and 4) appeared to retain the neo gene in 0.1% to 1% of marrow cells and (for dogs 3 and 4) activated T cells.

For dogs 2 through 5, we plucked individual CFU-GM, BFU-E, or CFU-Mix colonies obtained from marrow aspirates and assayed for the presence of neo (Fig 6). The percentage of positive colonies generally correlated with the level of marker retention obtained by visual densitometric estimates based on PCR amplification of DNA extracted from uncultured marrow cells (Table 3). For example, with dog 2, at 3 months posttransplant we found that 1 of 16 plucked CFU-GM was positive for neo, and this compared
favorably with the estimated 1% to 10% positivity based on amplification of DNA from uncultured marrow; at 13 months posttransplant, all 35 CFU-GM plucked and subjected to PCR amplification were negative, but analysis of DNA extracted from marrow cells indicated a level of positivity corresponding to that expected if about 0.1% of cells in the sample were genetically marked (Fig 5).

There was a sevenfold variation in the number of marked cells injected into the various dogs (Table 1). Dog 2, which still retained marked marrow cells at 21 months, received one of the largest doses of genetically marked cells. The data suggest that the proportion of marked marrow cells in MC+ dogs decreased from 1% to 10% over the first 1 to 4 months but then stabilized at ~0.1% to 1.0% after 3 to 6 months. Once cell dosages were taken into account, there was no detectable difference in the retention of neo-marked marrow cells between MC+ and MC− dogs up to at least 3 months after cell infusion.

Although helper virus was not detected in the PA317-N2 packaging line, it was important to conclusively establish that there was not virus spread in vivo. Plasma from the dogs was tested both for the presence of infectious neo virus and replication-competent helper virus. No virus was detected in either assay (Table 4).

**DISCUSSION**

One prerequisite for clinically relevant retrovirally mediated gene transfer into hematopoietic cells is the need to achieve highly efficient infection of reconstituting cells. LTMCs can provide access to pluripotent stem cells over an extended period of time in a contained environment, and therefore provide theoretical advantages in retroviral infection protocols. Previous attempts to achieve gene transfer into LTMC cells have involved exposures to retrovirus before long-term culture, or infection of LTMCs maintained for only 6 days. Hematopoietic growth factors have been used to augment in vitro gene transfer into hematopoietic cells, and preexisting adherent layers have been used to enhance culture viability.

In contrast to these approaches, we performed multiple infections of hematopoietic cells maintained in vitro in LTMCs for 3 weeks. Because retroviral vectors may require stimulation of cell proliferation for integration, and because LTMC progenitor cells are generally thought to be noncycling, we devised an infection strategy that was...
timed to coincide with the activation of cell proliferation thought to occur in LTMCs after media replenishment. We reasoned that an increasingly greater ratio of virus to cells at each week may also contribute to infection efficiency. With this approach and using a retrovirus bearing the gene for resistance to the neomycin analog G418 (neo),26,37 we obtained an average in vitro gene transfer efficiency into LTMC-derived CFU-GM that exceeded 70%.

The ultimate target of gene transfer into hematopoietic tissue is the pluripotent stem cell, which can only be indirectly assayed by actually determining its potential for hematopoietic reconstitution. There is evidence that such primitive progenitor cells are maintained in LTMCs.13 To assess the efficiency with which our reporter neo gene may have been transferred into this cell compartment, we transplanted the genetically marked LTMC cells into autologous recipients. All dogs have now been followed-up for 10 to 21 months and we have molecular genetic evidence that genetically marked myeloid and lymphoid hematopoietic cells and their progenitors are present in all five dogs (3 through 7) for which both compartments were examined, suggesting that primitive reconstituting progenitor cells were indeed infected by this protocol. Infusion of less than 10^6 infected CFU-GM/kg BW resulted in detectable engraftment of marked marrow progenitors for at least 21 months. In five dogs, marked marrow cells were retained for at least 1 year.

These results compare favorably with work by others on the transfer of foreign genes into canine cells involving either short-term cocultivation and selection,16,17 or cocultivation followed by supernatant infection of LTMCs maintained for 6 days.18,19 The in vitro gene transfer efficiencies obtained by these methods were not as high as we have achieved. Further, when infected cells were used in autologous transplants, the retention of infected cells was at a very low level and transient,18,20 even when in vivo selection was used.19,20

A second prerequisite for clinical implementation of

**Table 3. Detection of Neo Gene in Hematopoietic Cells After Autologous Transplantation of LTMC Cells**

<table>
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<th>Dog 2</th>
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Dogs 2 through 4 received marrow conditioning (MC+); dogs 5 through 7 received no marrow conditioning (MC−).

Abbreviation: ND, not done.

*Estimate of % BM cells genetically marked with neo as determined by PCR amplification of neo-specific sequences in DNA extracted from uncultured marrow and visual comparison to band intensities from amplifications of DNA in serial dilutions of positive controls.

†Number of positive colonies divided by total number of colonies tested, as determined by PCR amplification of neo-specific sequences in DNA extracted from individually plucked, non-G418-resistant CFU-GM, BFU-E, and/or CFU-Mix.

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aspirated from dog 2 at 13 months posttransplantation. This dog received 6Gy total body irradiation and was then injected with LTMC
cells from a dog that underwent marrow conditioning. Positive control consists of marrow cells from a dog that underwent transplantation without retrovirally infected cells.

retroviral-mediated gene transfer into hematopoietic cells in LTMCs is acceptable toxicity.3–4 Cocultivation and selection have been used to enhance gene transfer efficiency,4–6 but result in significant in vitro toxicity that compromises the growth of LTMCs and negates some of the advantages of using progenitors in LTMCs as targets for gene transfer. To explore better approaches, we modified standard LTMC techniques4–6 to maintain progenitor cells while achieving highly efficient infection. The infective retrovirus was prepared in LTMC media in an attempt to condition the media and further enhance culture viability. Omission of cocultivation steps prevented any potential loss of pluripotent stem cells due to adherence5 to virus producing fibroblasts. Omission of toxic selection by exposure to G418 permitted establishment of LTMCs with simple protocols uncomplicated by exhaustive marrow harvests, growth factors, or feeder layers. Perhaps a more intense infection schedule, with or without reduced duration of long-term culture, would result in an even greater efficiency of gene transfer without increased toxicity.

The role of marrow-ablative conditioning represents a third significant consideration in the clinical implementation of gene therapy addressed in this study. The results of previous studies suggest that the dosages of irradiation we used for marrow conditioning were high enough to be lethal in the absence of rescue by transplantation.3–6 In this study, however, dogs undergoing marrow conditioning received vigorous support including daily transfusion of irradiated blood products and comprehensive antibiotic coverage when necessary.3–6 This level of support permitted the hematopoietic recovery of dogs 8 and 9 after they received the conditioning regimen but no infusion of LTMC cells. For dogs that received the full transplant protocol, we therefore conclude that while some of the transplanted cells undoubtedly engrafted and contributed to recovery, the LTMC cells were not essential for recovery. In this context, it was surprising that we detected a significant number of genetically marked cells in vivo compared with the work of others.13–15 These results indicate that marrow progenitors maintained and retrovirally infected in LTMCs can engraft in the face of a presumably strong endogenous recovery of marrow during the pancytopenic postirradiation phase. They further indicate that the true frequency of transduction is unknown, as marrow conditioning was insufficient for ablation and potential factors influencing competition for recovery between endogenous and transduced stem cells are undefined at this time.

Omission of marrow conditioning did not result in any detectable reduction of in vivo retention of infected progenitors for 10 to 19 months posttransplant. This interesting observation suggests that LTMC cells can autologously engraft in the absence of marrow conditioning. Studies of syngeneic transplants in mice at higher cell dosages also indicate that marrow conditioning is not an absolute require-
ment for obtaining significant engraftment of autologous marrow. Similarly, immunocompromised mice do not need to be conditioned for reconstitution. Detection of engraftment after low cell dosages in this study may be related to the great sensitivity of gene detection made possible with PCR, or possibly the difference in species. These findings suggest that marrow conditioning and its associated toxicity may not be necessary for obtaining engraftment of genetically engineered cells. In the course of our on-going studies, we will determine how long the genetically marked cells are retained in the dogs that received no conditioning and further investigate how the interaction between marrow conditioning and cell dosage affects the efficiency of reconstitution with marked cells in this canine model.

In summary, we used multiple retroviral supernatant infections of day-21 LTMCs to obtain highly efficient gene transfer into canine progenitor cells. We then infused the marked culture cells into autologous recipients and showed that the culture cells engrafted into dogs with and without the use of marrow conditioning. Further, in at least one dog that received the complete transplant protocol, the inserted marker gene is still detectable in marrow cells 21 months after transplantation. Our method achieved gene transfer into a reconstituting stem cell population in a large mammal without the use of cocultivation or selection, growth factors, polycations, or other additional techniques. We conclude that this method represents a nontoxic and efficient method of gene transfer.

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REFERENCES

26. Bordignon C, Yu SF, Smith CA, Hanzopoulos P, Unger GE, Keever CA, O'Reilly RJ, Gilboa E: Retroviral vector-
mediated high-efficiency expression of adenosine deaminase (ADA) in hematopoietic long-term cultures of ADA-deficient marrow cells. Proc Natl Acad Sci USA 86:6748, 1989


44. Down JD, Tarbell NJ, Thames HD, Mauch PM: Syngeneic and allogeneic bone marrow engraftment after total body irradiation: Dependence on dose, dose rate, and fractionation. Blood 77:661, 1991


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