Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 350–354, January 1994
Medical Sciences

Gene transfer into hematopoietic stem cells: Long-term maintenance of *in vitro* activated progenitors without marrow ablation

(gene therapy/retrovirus/long-term marrow cultures/adoptive transfer)

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Communicated by Janet D. Rowley, September 3, 1993 (received for review May 24, 1993)

ABSTRACT **Adoptive transfer of genetically modified somatic cells will play an increasingly important role in the management of a wide spectrum of human diseases. Among the most appealing somatic cells as potential gene transfer vehicles are hematopoietic cells, because of their wide distribution and their well-characterized capacities for proliferation, differentiation, and self-renewal. Genes can be readily transferred into short-lived and lineage-restricted hematopoietic cells, but there remains a need to develop reliable methods for gene transfer into hematopoietic stem cells in large animals. In this work, we used a gene transfer approach in which hematopoietic cells in long-term marrow cultures were exposed to the replication-defective retrovirus N2, bearing the reporter gene *neo*, on multiple occasions during 21 days of culture. Genetically marked cultured autologous cells were infused into 18 canine recipients in the absence of marrow-ablative conditioning. *neo* was detected by Southern blotting and/or the polymerase chain reaction in the marrow, blood, marrow-derived granulocyte/macrophage and erythroid progenitors, and cultured T cells in dogs after infusion. In most dogs, the proportion of long-term marrow culture cells contributing to hematopoiesis rose during the first 3 months after infusion and peaked within the first 6. The maximal levels attained were between 10% and 30% G418-resistant (*neo*-positive) granulocyte/macrophage progenitors. At 12 months, five dogs maintained greater than 10% G418-resistant progenitors, and for two of them this level exceeded 20%. Two dogs had greater than 5% G418-resistant hematopoietic progenitors at 24 months after infusion. Our data suggest that very primitive hematopoietic progenitors are maintained in long-term marrow cultures, where they can be triggered into entering the cell cycle. *In vivo*, these activated cells likely continue normal programs of proliferation, differentiation, and self-renewal. Their progeny can be maintained at clinically relevant levels for up to 2 years without the requirement that endogenous hematopoiesis be suppressed through chemo- or radiotherapy prior to adoptive transfer. Long-term marrow culture cells may thus be ideal targets for gene therapy involving adoptive transfer of transduced hematopoietic cells.**

Replication-incompetent retroviruses are able to efficiently transfer exogenous genes into relatively mature clonogenic hematopoietic cells *in vitro* (1–5). Despite successes in this area, clinically relevant levels of gene transfer into nonmurine hematopoietic cells with long-term *in vivo* reconstituting capacities have not been achieved (6–11). A factor complicating attempts to transduce hematopoietic stem cells is the requirement that cells targeted for gene transfer must be cycling for stable integration of retrovirally carried genetic material (12). The vast majority of pluripotent hematopoietic stem cells are most likely quiescent *in vivo* (13, 14) and are thus difficult targets for gene transfer by retroviral vectors. Attempts to transduce pluripotent progenitors stimulated to enter the cell cycle during *in vitro* culture, with or without added growth factors, have given encouraging results (2, 4, 15–17). Similarly promising transduction efficiencies have been obtained by using hematopoietic cells harvested from animals in which hematopoiesis is in a postsuppression recovery phase (18–21). Approaches to identify, purify, and expand pluripotent stem cell *in vitro* for gene transfer applications have not been widely successful (22, 23). An important consideration in the adoptive transfer of transduced hematopoietic stem cells is the possibility that steady-state hematopoiesis may be maintained by the sequential use of a few stem cell clones, rather than through a static contribution of the entire stem cell pool (13, 14). If the vast majority of pluripotent hematopoietic stem cells are indeed quiescent *in vivo*, then it may be unlikely that genetically modified stem cells will be clonally expanded *in vivo*, once successfully engrafted.

We initiated studies to evaluate the potential of hematopoietic progenitor cells in long-term bone marrow cultures (LTMCs) to serve as targets for gene transfer. In LTMCs, an *in vitro* hematopoietic microenvironment is maintained for several weeks, during which proliferation of primitive hematopoietic cells occurs (24). We reasoned that these cells would be good targets for gene transfer for two reasons: (*i*) for large animals, LTMCs contain the most primitive hematopoietic cells characterized (23); and (*ii*) progenitor cells in LTMCs can be subjected to multiple rounds of retroviral infection while in culture (10). Furthermore, retrovirus-mediated gene transfer experiments directed at human and canine hematopoietic cells indicate that the efficiency of transduction is higher when progenitors in LTMCs are targeted than when hematopoietic cells are transduced directly in short-term culture (3, 5, 9, 10, 25, 26). Our results suggest that LTMCs contain an enriched population of primitive myelolymphopoietic cells that can be simultaneously transduced and activated *in vitro*. These cells have capacities for normal differentiation and proliferation programs *in vivo*, including self-renewal. Their progeny may be maintained at clinically relevant levels for up to 2 years in autologous recipients in the absence of marrow-ablative preconditioning.

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Abbreviations: BFU-E, erythroid burst-forming unit; CFU, colony-forming unit; CFU-GM, granulocyte/macrophage CFU; CFU-MIX, mixed erythroid-granulocyte/macrophage CFU; LTMC, long-term marrow culture.

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LTMC-derived hematopoietic stem cells may thus be ideal target cells for gene therapy involving adoptive transfer of genetically modified hematopoietic cells without requirements for *in vivo* selection and marrow-ablative conditioning.

MATERIALS AND METHODS

Dogs and Marrow Harvests. Dogs used in this study were obtained from the Central Animal Facility of the University of Guelph and were housed in the Veterinary Teaching Hospital until they recovered from all procedures. All protocols were approved by animal care, biohazard, and bioethics committees of the University of Toronto, The Toronto Hospital, and the University of Guelph. Marrow harvests were performed under general anesthesia from the iliac crests and proximal humeri and femora, as previously described (10, 27). LTMCs were established in the absence of preestablished feeder layers (10, 27).

Transduction of LTMC Cells. A cell-free supernatant of the packaging line PA317.N2, containing the retrovirus N2 (28), was used to transduce canine hematopoietic cells maintained in LTMCs. LTMC culture medium was as previously described (27). For the transduction protocol, LTMCs were initiated by inoculating Corning T-150 flasks (Corning) with 1.2×10^8 mononuclear cells in 60 ml of LTMC medium containing 1×10^6 virus per ml. On days 7 and 14 of incubation, the flasks were demidepopulated and the remaining cells were fed with non-virus-containing medium. On days 8 and 15, one-half of the supernatant layer of each flask was again removed and replaced with fresh medium containing 1×10^6 virus per ml. We reasoned that with this "21-day, three-cycle transduction protocol" primitive progenitor cells could be maintained in the adherent layers of LTMCs and triggered into cell cycling (29), thus providing the opportunity for optimizing retroviral transduction. After 21 days of culture, adherent cells were recovered by trypsinization and infused into the cephalic vein of the autologous recipients. Hematopoietic colony-forming units (CFUs) were assayed as previously described (10, 27). Evaluation of T cells was as previously described (10).

Assays of Gene Transfer Efficiency. Resistance to the neomycin analog G418 (GIBCO) was assessed by standard methods (10). For molecular genetic analyses, DNA from individual CFUs was extracted and subjected to PCR using primers specific for *neo*, the neomycin phosphotransferase gene (10). As an internal control to assess DNA quantity and quality, primers specific for an evolutionarily conserved region of the dystrophin gene were included in some PCR mixtures [a 23-mer complementary to the sense strand (5'-AATTCACAGAGCTTGCCATGCTG-3') and a 24-mer complementary to the antisense strand (5'-ACAGTCCTCTACTTCTTCCCACCA-3')].

Assays for Helper Virus and Clinical Follow-up. Plasma from reconstituted dogs was tested for the presence of infectious *neo*-bearing virus by using Rat 2 and 3T3 marker rescue assays, as previously described (10). Plasma was further assayed for the presence of helper virus by PCR using primers specific for the PA317 *env* gene (30). All dogs remained healthy for the duration of the study period and there was no evidence for the presence of replication-competent retrovirus.

RESULTS

Cell Harvests and Recoveries. The mean number of mononuclear marrow cells harvested was 6.1×10^7 cells per kg of body weight (BW) (range 7.5×10^6 to 1.4×10^8). Harvest volumes were 110–400 ml, representing 10–30% of estimated blood volumes. There were no complications associated with marrow harvests. The relative proportions of total nucleated cells aspirated, mononuclear cells recovered after density sedimentation, and clonogenic hematopoietic progenitors [erythroid burst-forming unit (BFU-E), granulocyte/macrophage CFU (CFU-GM), and mixed erythroid-granulocyte/macrophage CFU (CFU-MIX)] were within normal limits (10, 27). The dogs were divided into three groups based on the absolute number of day 21 LTMC cells infused. Group 1 dogs received 10^7–10^8 cells per kg BW; group 2 dogs, 10^6–10^7 cells per kg; and group 3 dogs, 10^3–10^4 cells per kg. Crude measures of progenitor cell contents of LTMC adherent layers were determined as 7871 (range 2604 to 13,870), 516 (range 80 to 819), and 1.2 (range 0.06 to 2.5) CFU-GM per kg BW for groups 1, 2, and 3, respectively (Fig. 1).

Efficient Gene Transfer into Committed Progenitors in LTMC. Mononuclear cells from day 21 LTMC adherent layers were plated in semisolid methylcellulose, and the ratios of hematopoietic colonies that grew in the presence of G418 to those that grew in control plates with no added G418 were determined. To further assess transduction, individual colonies from control and G418-containing plates were removed by using a finely drawn Pasteur pipette and subjected to PCR amplification using *neo* primers. The mean efficiency of gene transfer into CFU-GM derived from 3-week-old LTMCs was 44% when assayed by growth in G418 and 67% when assayed by PCR analysis of individual CFU-GM (Fig. 1).

LTMC-Derived Progenitors with Limited Self-Renewal and Extensive Proliferative Capacities Contribute to Hematopoiesis During the First Year after Infusion. *neo* DNA was detected in marrow and blood from all dogs during the first 12 months after infusion (Table 1). In 13 of 14 T-cell cultures established from 13 dogs within 15 months of infusion, *neo* was also detected (Table 1). There was wide variation among dogs with respect to the proportion of G418-resistant CFU-GM present during the first year after infusion of LTMC cells (Fig. 2). Generally, G418-resistant CFU-GM peaked in the 10–30% range during the first 3–6 months. There was no obvious difference between the three groups of dogs within this time period. CFU-GM scored as positive for growth in G418 were also positive for *neo* DNA by PCR.

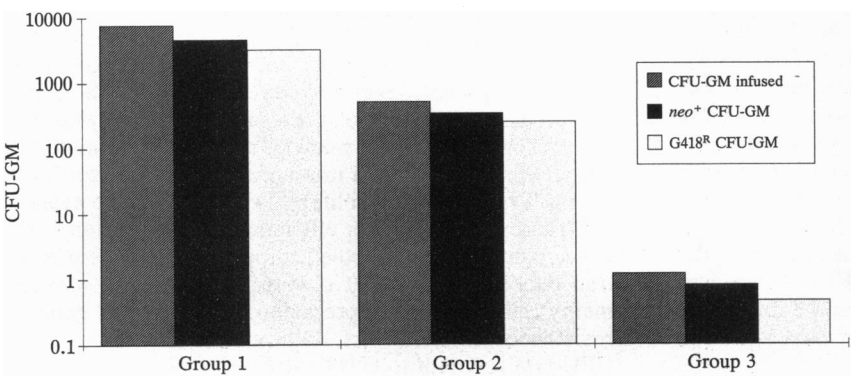

FIG. 1. Mean number of CFU-GM, *neo*-positive CFU-GM, and G418-resistant CFU-GM in day-21 LTMC cells infused into group 1, 2, and 3 dogs. There were eight dogs in group 1 and five each in groups 2 and 3. The numbers of *neo*-positive CFU-GM infused were extrapolated from the ratio of PCR *neo*-positive to PCR *neo*-negative CFU-GM in plates with no added G418. The number of G418-resistant CFU-GM infused was inferred by counting the CFU-GM present at day 10 in plates containing G418 at 1 mg/ml and CFU-GM in control plates with no added G418.

Table 1. Detection of *neo* in DNA from marrow (M), peripheral blood (Bl), CFU-GM and/or BFU-E (CFU), and activated T-cells (T) after infusion of transduced LTMC cells

	Dog 1				Dog 2				Dog 3				Dog 4				Dog 5				Dog 6				Dog 7				Dog 8			
Mo.	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T
1			+				+				+								+													
3		-	+				+			+	+			+					+								+					
6	+*	+	+		+*	+	+		+*		+		+	-			+	+	+			+	+			+	+					
9	+	+		+	+			+	+	-			+	+			+			+	+*			+	+				+			+
12			+				+				+				+				+				+				+	+			+	
15															+	+																
18							+							-																		
21																					+		+									
24													-	-											+		+	-	+		+	

Group 2

	Dog 9				Dog 10				Dog 11				Dog 12				Dog 13			
Mo.	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T
1											+				+				+	
3		+				-	+			-	+				+			-	+	
6	+*	-	+		+*	-	+	+	+	-	+		+	+	+		+	-	+	
9	+	+		+	+								+			+	+			+
12			+				+								+					
15															+				+	
18																				
21	+		+																	

Group 3

	Dog 14				Dog 15				Dog 16				Dog 17				Dog 18			
Mo.	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T	M	Bl	CFU	T
1			+				+				+				+				+	
3		+	+			-	+			-	+			-	+			-	+	
6	+	+	+		+	+	+		+*	+	+		+*	-	+		-	-	+	
9		+			+				+				+	+		+				-
12	+		+	+							+				+				+	
15	-	-							+		+	+								
18											+									

neo-positive results are indicated by + and negative results by −; blanks indicate times for which no data are available due to insufficient follow-up, the unavailability of samples, or inconclusive PCR data. All samples were assayed by PCR. Samples also assessed by Southern blotting are indicated by +*.

LTMC-Derived Progenitor Cells with Extensive Self-Renewal Capacities Proliferate and Differentiate *in Vivo*. Marrow and blood samples were studied between 1 and 2 years after infusion of LTMC cells. In all 16 dogs studied in this time period, *neo* DNA was detected by PCR analysis of individual CFU-GM and/or marrow, blood, or cultured T-cells (Fig. 2 and Table 2). For the five dogs receiving the least number of LTMC cells (group 3) the proportion of G418-resistant CFU-GM declined to less than 1% by 15 months (Fig. 2*C*). For three of five dogs in group 2, receiving 10^2 to 10^3-fold more LTMC cells, the results were similar. However, for the remaining group 2 dogs, 21% G418-resistant CFU-GM were detected at 15 months after infusion in one (no. 13) and 14% at 21 months in the other (no. 9) (Fig. 2*B*). For the dogs receiving the greatest number of LTMC cells (group 1), the decline in the proportion of G418-resistant CFU-GM was less pronounced than that observed for group 2 and 3 dogs (Fig. 2*A*). Three of the eight dogs in group 1 maintained about 5% G418-resistant CFU-GM at 21–24 months.

DISCUSSION

In an attempt to develop improved methods for gene transfer into hematopoietic stem cells, canine LTMCs were exposed to the replication-defective retrovirus N2, bearing the reporter gene *neo*, at culture initiation and on days 8 and 15 during 21 days of culture. Widely different numbers of transduced autologous LTMC cells were infused into 18 healthy recipient dogs. Dogs received no marrow ablative conditioning whatsoever and were divided into three groups based on the numbers of cells infused. Gene transfer and expression in relatively mature, clonogenic, hematopoietic progenitors as well as in more primitive progenitors with *in vivo* self-renewal and proliferative capacity were evaluated.

To assess gene transfer efficiency and expression in short-lived committed hematopoietic progenitors, adherent layers of transduced day-21 LTMCs were harvested and assayed in methylcellulose. For expression of the reporter gene, *neo*, resistance to G418 was determined. The proportion of CFU-GM resistant to G418 exhibited wide variation about a

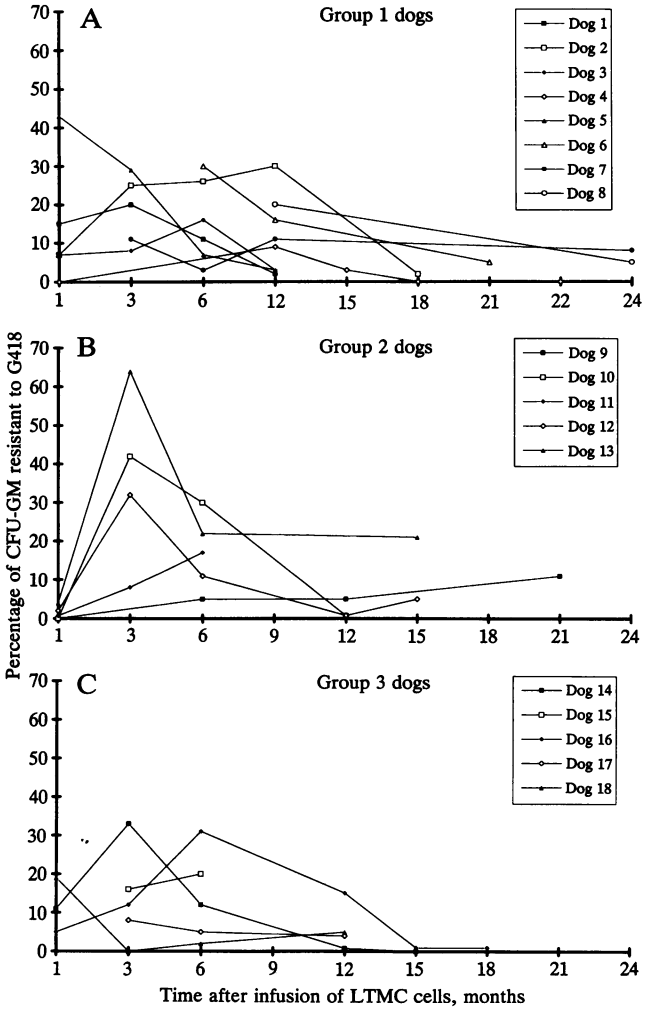

FIG. 2. Percentage of CFU-GM resistant to G418 for group 1, 2, and 3 dogs (*A*, *B*, and *C*, respectively) at indicated times after infusion of LTMC cells. The percentage of G418-resistant CFU-GM infused was determined by comparing the numbers of CFU-GM present at day 10 in plates containing G418 at 1 mg/ml to the numbers of CFU-GM in control plates with no added G418. Each assay was done in quadruplicate.

mean of 44%. The mean gene transfer efficiency into CFU-GM, as measured by PCR amplification of *neo*-specific sequences, was approximately 67%, and it ranged from 30% to ≈100% (Fig. 1). The *in vitro* gene transfer efficiency into

Table 2. Detection of *neo* in myeloid and lymphoid lineages in dogs positive beyond 12 months after infusion of LTMC cells

Dog	Time after infusion, mo	*neo* (PCR analysis)		$G418^R$ CFU
		Myeloid	Lymphoid	
2	18	+		+
4	15	+	+	+
6	21	+		+
7*	24	+	−	+
8	24	+		+
9	21	+		+
12	15	+		+
13	15	+		+
16*	15	+	+	+

Positive results are indicated by + and negative results by −; blanks indicate times for which no data are available.

*Dogs 7 and 16 were positive for *neo* in myeloid and lymphoid cells at 12 months. Dog 16 remained positive at 15 months in both lineages, but dog 7 remained positive in myeloid cells only at 24 months.

short-lived committed hematopoietic progenitors obtained in these studies is consistent with that achieved by other investigators working with hematopoietic cells from large animals (1–5, 9).

To evaluate the *in vivo* hematopoietic reconstituting potential of LTMC cells, *neo*-marked day-21 autologous LTMC cells were infused into recipient dogs. To specifically assess repopulating potential in the presence of competition from endogenous stem cells, recipient dogs were not subjected to any type of marrow conditioning and were infused with limiting dilutions of transduced LTMC cells. In all dogs after infusion, marrow-derived CFU-GM, BFU-E, and CFU-MIX grew in the presence of G418 at toxic concentrations. The mean percentage of CFUs resistant to G418 at 6 months after infusion was ≈15%. This figure declined to 6.5% and 6% at 12 and 15 months, respectively. At 9–15 months fresh marrow cells and cultures of activated T cells were *neo* positive by PCR analysis. In the longest-followed dogs, LTMC-derived progenitors still contributed to hematopoiesis (≈5%) at 24 months after infusion (Fig. 2*A*). Most significantly, CFU-GM derived from 21-day LTMCs established with postinfusion marrow were G418 resistant at 12–21 months in 14 of 15 dogs studied. Furthermore, while the five dogs for which G418-resistant CFUs were detected at 5–30% between 1 and 2 years after infusion were among those that received the largest numbers of LTMC cells, dogs that received 1/100th to 1/1000th as many cells also showed comparable levels of G418-resistant CFUs up to the 12 month time point (Fig. 2*C*).

Our results embody the longest follow-up data of which we are aware on retention of transduced hematopoietic cells and reporter gene expression in a large animal species (6–9, 11, 31–33). Our observations demonstrate that relatively small numbers of hematopoietic progenitor cells can give rise to relatively large numbers of differentiated progeny in the *in vivo* hematopoietic microenvironment in the absence of selection and marrow ablative conditioning. Our results further indicate that some progenitors in LTMCs have capacities for self-renewal and long-term proliferation *in vivo* and that they can be readily transduced by using the 21-day, three-cycle, transduction protocol described herein. To account for these findings we suggest the following: In the first instance, it is likely that hematopoietic stem cell quiescence under steady-state hematopoiesis (13, 14) is at least partially maintained by physiological feedback inhibition mechanisms in which mature blood elements play a major role. In LTMCs, the relative proportion of mature hematopoietic cells to primitive progenitors is significantly reduced, and we postulate that under these conditions physiological mechanisms favor activation of quiescent stem cells. Second, in our protocol, exposure to retrovirus was timed to occur when the general cell cycling stimulatory effects of LTMC media replenishment are likely most operative (29). We suggest that these two factors enhanced transduction of normally quiescent stem cells. Not surprisingly, when one or both of these steps are omitted the efficiencies of stem cell transduction are lower (6–11, 31–33).

An important observation made in this study is that LTMC-derived cells are capable of contributing significantly to hematopoiesis *in vivo* in the presence of competition from endogenous stem cells. None of the dogs in this study were subjected to any type of marrow-ablative conditioning, yet relatively high levels of G418-resistant CFUs were detected in all dogs. While the levels of engraftment observed here in the absence of marrow-ablative conditioning may seem surprising, there are precedents for this. Murine studies indicate that marrow conditioning is not an absolute requirement for obtaining significant engraftment of marrow (34, 35). There is also good evidence from studies of chimerism after human therapeutic bone marrow transplantation to support the idea that 100% marrow ablation is not essential for engraftment (36). The recent report of donor-derived long-term multilin-

eage hematopoiesis in a liver transplant recipient (37) further supports this idea. Our data thus suggest that the widely held concept that creation of marrow "space" by irradiation is essential for engraftment may be invalid. Indeed, one effect of marrow ablation prior to adoptive transfer may be the activation of residual stem cell cycling in the host, and this may help explain why gene transfer using the approach outlined here yielded results better than those obtained when marrow-ablative preconditioning was used (6–11, 31–33).

Our results also demonstrate that the relative contribution to overall hematopoiesis made by transduced LTMC cells is far greater than would be expected on the basis of the numbers of cells originally infused. To account for this, we suggest that progenitor cells activated in LTMCs continue normal proliferative programs *in vivo*. We further suggest that one physiological response to an excess of cycling stem cells may be to simply permit actively proliferating cells to complete their normal differentiation programs until homeostasis is attained, rather than to actively shift some stem cells to quiescence. The data of Collins *et al.* (37), discussed above, support this concept of a passive physiological response to activated stem cells. Furthermore, Spain and Mulligan (21) recently demonstrated that when 5-fluorouracil-activated partially purified stem cells are infused into mice the transduced stem cells similarly contribute to *in vivo* hematopoiesis to a greater extent than would be predicted from their low frequency.

In summary, in the course of optimizing our retroviral transduction procedure to target quiescent and primitive hematopoietic cells, we apparently succeeded in stimulating primitive progenitors in LTMCs into sustained, active cell cycling. This was possibly a physiological response to the overall *in vitro* cytoreduction achieved by weekly demidepopulation and media replenishment of cultures. These activated progenitors continued to proliferate and give rise to differentiated progeny when infused into unirradiated autologous recipients. Most of these "*in vitro* activated" hematopoietic progenitors underwent clonal extinction during the first 12 months *in vivo*. However, some clearly underwent extensive self-renewal *in vivo*, and their multilineage progeny were maintained for up to 2 years. It thus appears that pluripotent hematopoietic stem cells in LTMCs may be simultaneously transduced and activated *in vitro* and subsequently undergo normal differentiation programs *in vivo*, including self renewal. Such cells may be ideal targets for gene therapy in settings in which *in vivo* selection is not practical and marrow-ablative conditioning is undesirable.

Note Added in Proof. While this manuscript was under review, two papers supporting the concept that donor marrow can successfully and permanently repopulate a recipient mouse without the need for removing or damaging recipient hematopoiesis were published (38, 39).

We thank R. A. Phillips, C. J. Eaves, and G. B. Mills for helpful discussions and for reviewing the manuscript and P. N. Ray for the dystrophin primers. R.F.C. was the recipient of a Fellowship from the Medical Research Council of Canada. The research in the laboratories of J.E.D., I.D.D., and S.K.-R. are supported by the Medical Research Council and the National Cancer Institute of Canada. This work was supported by Medical Research Council of Canada Grant MT10259 to I.D.D., S.K.-R., S.A.K., and A.C.G.A.-O.

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