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Long-term *in vivo* expression of the human glucocerebrosidase gene in nonhuman primates after CD34$^+$ hematopoietic cell transduction with cell-free retroviral vector preparations

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ABSTRACT **Successful gene transfer into stem cells would provide a potentially useful therapeutic modality for treatment of inherited and acquired disorders affecting hematopoietic tissues. Coculture of primate bone marrow cells with retroviral producer cells, autologous stroma, or an engineered stromal cell line expressing human stem cell factor has resulted in a low efficiency of gene transfer as reflected by the presence of 0.1–5% of genetically modified cells in the blood of reconstituted animals. Our experiments in a nonhuman primate model were designed to explore various transduction protocols that did not involve coculture in an effort to define clinically useful conditions and to enhance transduction efficiency of repopulating cells. We report the presence of genetically modified cells at levels ranging from 0.1% (granulocytes) to 14% (B lymphocytes) more than 1 year following reconstitution of myeloablated animals with CD34$^+$ immunoselected cells transduced in suspension culture with cytokines for 4 days with a retrovirus containing the glucocerebrosidase gene. A period of prestimulation for 7 days in the presence of autologous stroma separated from the CD34$^+$ cells by a porous membrane did not appear to enhance transduction efficiency. Infusion of transduced CD34$^+$ cells into animals without myeloablation resulted in only transient appearance of genetically modified cells in peripheral blood. Our results document that retroviral transduction of primate repopulating cells can be achieved without coculture with stroma or producer cells and that the proportion of genetically modified cells may be highest in the B-lymphoid lineage under the given transduction conditions.**

Somatic cell gene therapy has been used in the treatment of adenosine deaminase (ADA) deficiency, and clinical trials for other disorders are ongoing (reviewed in refs. 1–3). Gaucher disease, an autosomal recessive lysosomal storage disorder, is characterized by a genetic deficiency of glucocerebrosidase (GC) and an accumulation of glucocerebrosides in bone marrow-derived macrophages (4). Enzyme replacement therapy (5–7) or allogeneic bone marrow transplantation (8, 9) offers therapeutic alternatives, but enzyme replacement therapy requires repetitive intravenous treatment, and allogeneic bone marrow transplantation is associated with severe morbidity and significant mortality (10). Development of gene replacement for Gaucher disease has therefore assumed a high priority. The human GC gene has been transferred into hematopoietic progenitors from patients with Gaucher disease, with correction of the enzyme deficiency (11–13). Hematopoietic stem cells (HSC) of mice (14–19) can also be efficiently transduced. The human GC gene has also been transduced into human CD34$^+$ cells, which have repopulated immunocompromised mice for long periods of time (20). Expression of human GC has been documented in macrophages of long-term reconstituted mice transplanted with retrovirally transduced HSC (17, 18).

Successful gene transfer into HSC of larger animals, including nonhuman primates, has been achieved by using protocols involving coculture with retroviral producer cells (21), autologous stroma (22), or an engineered murine stromal cell line expressing human transmembrane stem cell factor (SCF) (23). After reconstitution, only 0.1–5% of cells of various lineages contained the proviral genome. Expression of a transferred ADA gene has been documented >1 year after transplantation (23). The present experiments were designed to explore various conditions for transduction of HSC that did not involve coculture with foreign cells in an effort to enhance both transduction efficiency and develop methodology that could be applied clinically. Persistent expression of the recombinant human GC gene has been documented in hematopoietic cells of primate transplant recipients.

MATERIALS AND METHODS

Animals. Young adult rhesus macaques (*Macaca mulatta*) used in these studies were serologically negative for simian T-cell lymphotropic virus (STLV), simian immunodeficiency virus (SIV), and simian AIDS-related type D virus (SRV). Animals with blood type B were selected and had an indwelling central catheter established. Experimental animals were quarantined and housed in accordance with the guidelines set by the Committee on Care and Use of Laboratory Animals (24) and the policies set by the Veterinary Research Program of the National Institutes of Health. The protocols evaluated were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute.

Vector Production. The organization of the vector containing the GC gene is shown in Fig. 1. Derivation of a high-titer amphotropic producer clone (LG4) by repeated infection of the GP+envAm12 cell line with medium conditioned by an ecotropic retroviral producer line has been described (13). This amphotropic producer clone was devoid of replication-competent viruses as documented with a marker rescue assay (25, 26). Twelve to 14 hr after refeeding the producer cells at

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Abbreviations: ADA, adenosine deaminase; BM, bone marrow; GC, glucocerebrosidase; HSC, hematopoietic stem cell(s); IL, interleukin; MN, mononuclear; PB, peripheral blood; RT, reverse transcription; SCF, stem cell factor.

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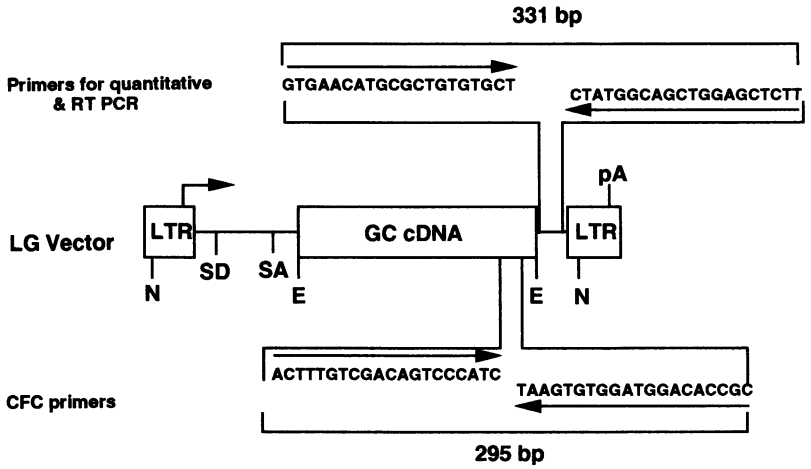

FIG. 1. Retroviral vector used for GC cDNA transfer. LG, a murine retrovirus-based vector that includes the GC cDNA driven by the Moloney murine leukemia virus long terminal repeat (LTR). SD, splicing donor; SA, splicing acceptor; pA, polyadenylylation site; E, *Eco*RI; and N, *Nhe* I. The arrow above LTR indicates the transcriptional start site and direction of transcription. PCR primers and sizes of the expected PCR products (bp) are indicated. Arrows above the primer sequences indicate the direction of 5′-to-3′ DNA synthesis. CFC, colony-forming cell. The diagram is not proportional to actual size.

90–100% confluency with the Dulbecco-modified Eagle medium containing 15% (vol/vol) fetal bovine serum, viral supernatant with an estimated titer of 10^8 particles per ml was harvested and used immediately without freezing. Virus titer was determined as described (12) on 3T3 cells lacking thymidine kinase.

Transduction of Primitive Hematopoietic Cells. $CD34^+$ cells were recovered by positive immunoselection of bone marrow (BM) mononuclear (MN) cells as described (22, 27). A 15- to 70-fold enrichment in clonogenic progenitors was achieved. For viral transduction, $CD34^+$ cells were cultured at a density of 2×10^5 cells per ml in fresh viral supernatant harvested from the amphotropic producer cells in the presence of 2 mM L-glutamine, protamine at 4 μg/ml, interleukin 3 (IL-3) at 10 ng/ml, interleukin 6 (IL-6) at 50 ng/ml, and SCF at 100 ng/ml (provided by Amgen Biologicals). The cells were refed daily with the fresh supernatant for 4 days prior to harvesting and were reinfused as described (22). A 3- to 5-fold amplification of total cell number was achieved during *ex vivo* culture. Cytospin preparations of the cells prior to and after culture were made to document cellular morphology. For two animals, an autologous stromal cell layer was established by serial passage of adherent cells recovered from a BM aspirate as described (22). A 10- to 20-fold expansion of the $CD34^+$ population was achieved over 7 days in a 25-mm tissue culture insert (Nunc Roskilde) that separated the $CD34^+$ cells from autologous stroma; IL-3, IL-6, and SCF were added at concentrations of 10, 50, or 100 ng/ml, respectively. The expanded cells were then transduced as described above either with (animal RQ383) or without (animal RQ234) reselection of $CD34^+$ cells from the expanded cell population prior to transduction. Transduced cells were assayed for clonogenic activity by plating 10^3–10^4 cells in 1 ml of HCC-4200(s) Iscove's methylcellulose (Terry Fox Laboratories, Vancouver) supplemented with 2 mM L-glutamine and 10 ng of granulocyte/macrophage-colony stimulating factor per ml in a 35-mm culture dish. Isolated colonies were picked for detection of the proviral genome by polymerase chain reaction (PCR) analysis (13). The primers used for PCR amplification of the isolated colonies are the colony-forming cell primers shown in Fig. 1.

BM Transplantation. The protocol was as described (22) with the following modifications. On day −2 and day −1, the animal received 650 R (dose rate: 8.8 R/min) total body γ-irradiation. On day 0, the animal received transduced, autologous $CD34^+$ cells (in 50 ml of Hanks' balanced saline solution containing 1% fetal calf serum and 10 units of heparin per ml). Standard supportive care, including infusion of granulocyte-colony stimulating factor (5 μg/kg of body weight per day), was initiated following bone marrow transplantation.

Cell Purification. MN cells from peripheral blood (PB) were collected following density centrifugation. Granulocytes (95–98% pure) were recovered by lysis of erythrocytes in the pellet with ammonium chloride after a second purification by density centrifugation. For some experiments, further purification of granulocytes was achieved by flow cytometry sorting based on size (forward light scatter) and granularity (90°-angle light scatter). T or B lymphocytes were isolated from the MN cells by cell sorting. Fluorescein 5-isothiocyanate-conjugated CD2, phycoerythrin-conjugated CD20, and their isotype-matched controls were obtained from Coulter. The concentration of antibody used was in accordance with the manufacturer's instructions. After staining, the cells were washed twice in cold phosphate-buffered saline supplemented with 1% bovine serum albumin. The cells were sorted on a Coulter Elite flow cytometer. Sorting purity was 99% or better.

Quantitative PCR Analysis. DNAs from PB and BM MN cells and from granulocytes were isolated by standard techniques (13, 22). To quantitate the efficiency of gene transfer of the LG vector into hematopoietic cells of rhesus primates, dilutions of DNA from a packaging cell line (PA317-LG11) containing a single copy of the provirus per cell were used as standards. The vector sequences were detected by PCR amplification of 800 ng of genomic DNA from the PB MN, BM MN, or granulocytes as a template with specific primers (Fig. 1) (forward, GTG AAC ATG CGC TGT GTG CT; reverse, TTC TCG AGG TCG ACG GTA TC). Equivalent loading of DNA was demonstrated by using rhesus gamma globin primers (forward, GTT GGG AGT GAA GAA ACT GC; reverse, TAG CCT CAG ACT CTG TTT GG). Conditions used for PCR amplification were as follows: initial denaturation at 94°C for 5 min; a total of 30 cycles of amplification, each cycle with denaturation (at 93°C for 1.5 min), annealing (at 65°C for 2 min), and elongation (at 71°C for 1.5 min with a 2-sec extension in each additional cycle); a final reaction at 65°C for 4 min and 71°C for 8 min; and then a slow cooling to room temperature. Amplified products were loaded on 1% agarose gels. At the end of electrophoresis, the gel was illuminated on UV and blotted onto Nytran membrane for Southern blot analysis with ^{32}P-labeled GC cDNA probes.

The vectors were also detected by [α-^{32}P]dCTP PCR using 200 ng of DNAs from the flow cytometry-sorted T or B lymphocytes or granulocytes as a template, with the primers

and conditions described above except that 0.3 μl of [α-^{32}P]dCTP (800 mCi/mmol; Amersham) was added to each reaction. The amplified products were visualized by phosphor screen autoradiography using a PhosphorImager (Molecular Dynamics). Bands were quantitated by volumetric integration by ImageQuant.

Reverse Transcription (RT)–PCR. MN cells from the PB were collected following density centrifugation. Total cellular RNAs were isolated using TRIzol reagent (GIBCO/BRL) according to manufacturer's instructions. RNA samples were further treated at 37°C for 20 min with a cocktail containing 50 mM Tris chloride (pH 7.4), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 26 units of RNase inhibitor (Promega) and 6 units of DNase I (Boehringer Mannheim) per 50-μl reaction volume to remove possible contamination of the sample with DNA. The first-strand cDNA was synthesized with 0.8 μg of the total RNA as a template with the reverse primers specific to the GC of the LG vector (Fig. 1). Twenty units of avian myeloblastosis reverse transcriptase (Boehringer Mannheim) was used per 20-μl reaction volume, and reaction conditions were according to the manufacturer's instructions. The cDNA was amplified by using AmpliTaq from Perkin–Elmer/Cetus. PCR conditions and analysis of amplified products were the same as described above in the quantitative PCR analysis.

RESULTS

Transduction of Rhesus Primitive Hematopoietic Cells with the Retroviral Vector Containing the GC Gene. During *ex vivo* culture for 4 days, the total number of cells increased 3- to 4-fold. Thirty to sixty percent of clonogenic progenitors were transduced as documented by detection of retroviral sequences in colonies grown in semisolid medium (Table 1). Extension of the *ex vivo* culture to 7 days over autologous stroma resulted in a 10-fold expansion in cell numbers; after 4 additional days of culture with retroviral vector, prominent myeloid differentiation was observed (animal RQ234). Minimal myeloid differentiation was observed when the expanded cells at 7 days were reselected for CD34$^+$ cells (animal RQ383). This reselection for CD34$^+$ cells prior to transduction resulted in a loss of 82% of the total number of cells present in culture. The number of cells reinfused for each animal is shown in Table 1. For those animals receiving total body irradiation, the day when the leukocyte count reached 1000 cells per μl varied from 12 to 35. One animal, RQ234, remained transfusion dependent until it was euthanized on day 63. CD34$^+$ cells from this animal had been cultured for 7 days in tissue culture inserts and then transduced for 4 additional days without prior CD34$^+$ reselection. The animal experienced significant respiratory distress immediately upon reinfusion of the expanded cells. Two animals (92T and 9AB) were transplanted with transduced autologous CD34$^+$ bone marrow cells without myeloablation.

Detection of Genetically Modified Cells in Hematopoietic Tissues. As summarized in Table 1, the PB cells of primates RQ352, RQ398, and RQ383 contained the proviral genome when tested at approximately 3, 6, 12, and >15 months after bone marrow transplantation. Animal RQ234 was vector positive in the PB and the BM when euthanized on day 63. The nonablated animals (92T and 9AB) had low numbers of genetically modified cells in both PB and BM after transplantation, but the PCR analysis became negative when tested on day 116 (92T) and day 56 (9AB) (Table 1) and has remained negative ever since.

Fig. 2 shows semiquantitative PCR analysis of the LG vector in the PB MN cells, BM cells, and PB granulocytes from RQ352 on day 537, RQ398 on day 529, and RQ383 on day 461. The standard titration was done using serial dilutions of the genomic DNA of 3T3/LG11 cells (containing a single copy of the provirus per cell) with DNA isolated from a negative, nontransplanted primate so that a total of 200 ng of DNA was maintained per lane. The specific GC signal was easily detectable at a dilution of 1:200 in this experiment. As shown in Fig. 2, the density of the PCR-amplified product when using 200 ng of the genomic DNA of the PB MN (Fig. 2*A*) as templates was at least equal to that of the 1.9% dilution of the DNA of the 3T3/LG11 cells. This result indicates that at least 1% of the PB MN cells in RQ352, RQ398, and RQ383 contained the LG vector. To further characterize the LG-marked cell populations, the PB MN cells were sorted by flow cytometry into B or T lymphocytes based on CD20(B1) or CD2(T11) expression, respectively. Granulocytes were sorted by flow cytometry on forward light scatter (size) and 90°-angle light scatter (granularity). DNA from each lineage was isolated and analyzed by [α-^{32}P]dCTP PCR and phosphorImager quantitation. Based on results shown in Fig. 2*A*, the percentage of B-cell marking ranged from 1% to 14%, and the range of T cells containing LG vector was 0.3%–3%. Granulocytes were marked by the LG vector at a lower frequency, ranging from 0.1% to 0.5%. At a later date, PB, BM, and a popliteal lymph node were taken from the animals, and PCR analysis of these tissues showed respectively 2–4%, 4–16%, and 12–22% GC gene marking (Fig. 2*B*).

Table 1. Characteristics of retroviral supernatant transduction of BM CD34$^+$ cells of rhesus primates and outcome of autologous BM transplantation

Animal	Total body γ-irradiation	Transduction conditions	Transduction efficiency	Cells infused,* no.	Days WBC > 1000†	Clinical + PCR status‡
RQ352	6.5 Gy × 2	Supernatant	6/10	1.5×10^8 (3.8×10^7/kg)	15	Alive PCR + (D 608)
RQ398	6.5 Gy × 2	Supernatant	6/10	1.0×10^8 (2.8×10^7/kg)	12	Alive PCR + (D 595)
RQ383	6.5 Gy × 2	Transwell CD34 reselection, + supernatant	5/10	7.0×10^7 (1.8×10^7/kg)	28	Alive PCR + (D 531)
RQ234	6.5 Gy × 2	Transwell + supernatant	3/10	1.9×10^8 (4.1×10^7/kg)	35	Euth. D 63 (graft failure)
92T	None	Supernatant	4/10	3.9×10^7 (8.8×10^6/kg)	NA	Alive PCR − (D 116)
9AB	None	Supernatant	5/10	4.5×10^7 (1.6×10^7/kg)	NA	Alive PCR − (D 56)

*Numbers in parentheses relate the number of cells infused per kg of animal body weight.
†NA, not applicable; WBC, leukocytes.
‡All animals were evaluated ≈30 and 90 days after transplant and every 3 months thereafter. RQ352, RQ398, and RQ383 were tested positive at all time points and remained positive until the last test date indicated above. 92T and 9AB were no longer positive after the indicated days (D). Euth, euthanized.

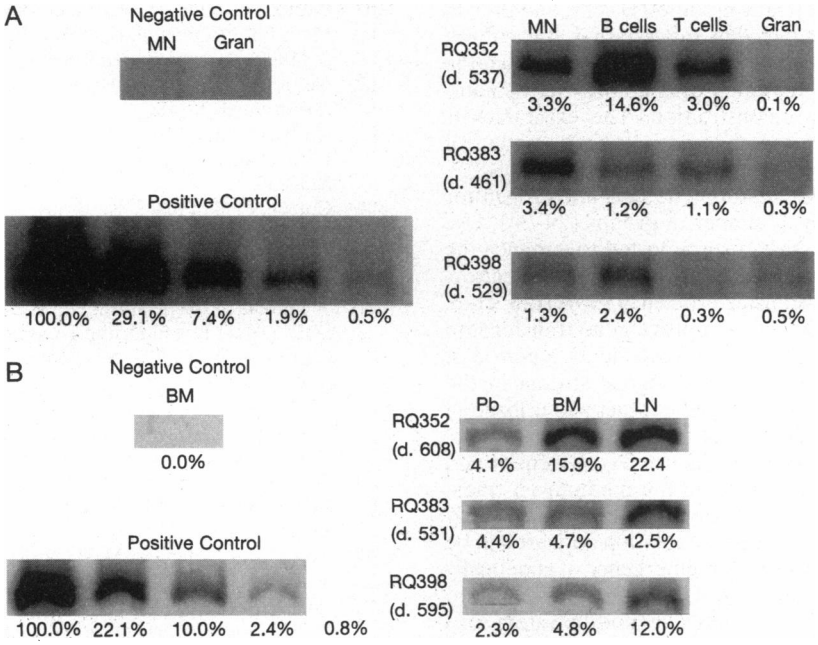

FIG. 2. Quantitation and lineage analysis of gene transfer into hematopoietic cells of rhesus monkeys. (*A*) DNA samples from MN cells of PB leukocytes were isolated and used for quantitative PCR analysis with PCR primers from the retroviral backbone downstream from the GC cDNA sequence (Fig. 1). MN cells of PB were isolated and sorted into B lymphocytes and T lymphocytes based on the characteristic expression of CD20 (B1) and CD2 (T11), respectively. Granulocytes were isolated by using flow cytometry as described in text. DNA from each lineage was isolated and analyzed by PCR and PhosphorImager quantitation. The resulting band density for each sample was compared to those of a known DNA standard. The samples from RQ352, RQ398, and RQ383 were evaluated on days 537, 529, and 461 after gene transfer, respectively. (*B*) PB, BM, and popliteal lymph nodes (LN) were isolated and analyzed by using PCR and PhosphorImager quantitation.

Expression of the Transferred GC Gene. To evaluate whether the human GC gene was expressed, RT-PCR was used to detect the GC mRNA from PB MN cells of RQ352 on day 565, of RQ398 on day 489, and of RQ383 on day 552. Lanes 2–4 in Fig. 3 show the RNA amplified without RT, and lanes 6 to 8 show the RNA amplified with RT using the reverse primers specific to the LG vector. Lanes 1 and 5 represent a RNA sample from a negative control animal. The RT-PCR methodology successfully detected the GC mRNA from the PB MN cells.

DISCUSSION

High efficiency of gene transfer into hematopoietic stem cells in human and large animal models has been more difficult to achieve than in murine systems (1, 21–23). van Beusechem *et al.* (21) demonstrated coculture genetic modification of rhesus monkey hematopoietic cells (10–100 provirus genomes per 10^5 BM cells, equivalent to 0.01–0.1%) 6 months after autologous transplantation of retrovirus-infected BM cells and demonstrated expression of functional human adenosine deaminase in PBMN cells. By coculturing nonhuman primate immunoselected $CD34^+$ BM cells on a stromal cell line engineered to express the transmembrane form of SCF, we were able to detect a phosphoglycerate kinase (PGK)-ADA provirus in 0.75% to 5% (an average of 2%) of the PB cells >1 year after autologous transplantation of transduced cells (23). Murine ADA activity was also detected in the PB cells, having a level of activity ≈3% of that of the endogenous primate ADA enzyme. Experiments in canine models using cells cocultured with autologous stroma have given similar numbers of genetically modified cells after transplantation. We have now established that cells capable of contributing to long-term restoration of hematopoiesis can be transduced with retroviral vectors in suspension culture without contact with a stromal or producer monolayer. Analogous results have been obtained in pediatric-age patients who were subjected to autologous transplantation with transduced BM cells, harvested after several cycles of myelosuppressive chemotherapy, given for treatment of a malignancy (28).

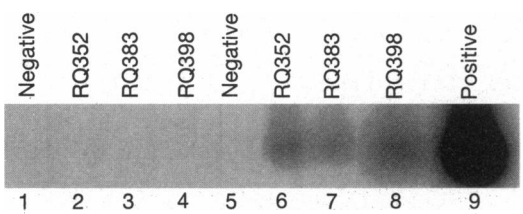

FIG. 3. Determination of expression of transferred GC gene in hematopoietic cells of rhesus monkeys by RT-PCR analysis. MN cells of PB were isolated, and RNA was prepared for RT-PCR by using LG primers specific for the recombinant GC sequence. Standard techniques were used for Southern analysis of RT-PCR product. Lanes: 1 and 5, RNA from a negative control animal; 2–4, RNA samples amplified in the absence of reverse transcriptase; 6–8, RNA samples amplified in the presence of reverse transcriptase; and 9, DNA isolated from the LG producer cell line. The RNA sample was taken from RQ352 on day 565, from RQ383 on day 489, and from RQ398 on day 552.

Two to four percent of the circulating leukocytes from the transplanted primates contained the GC provirus based on PCR analysis. Interestingly, 4–16% of the cells from the BM and 12–22% of the cells from the popliteal lymph nodes contained the GC provirus. The reason for this discrepancy was not resolved but may have been due to a lymphocytosis within the primate BM, since the GC provirus was detected in a greater proportion of B cells than T cells or granulocytes, and/or due to a selective loss of circulating granulocytes containing the human GC gene.

An interesting facet of our results is the relatively high frequency of genetically modified B and T lymphocytes compared with myeloid cells in reconstituted recipients. Transduction of $CD34^+$ cells during coculture with a murine stromal cell line expressing human transmembrane SCF resulted in a

delayed emergence of genetically modified B cells, and then at very low frequency. These results suggest that the culture conditions to which stem cells are exposed may influence the distribution of genetically modified cells among the hematopoietic lineages following reconstitution. The exact factors responsible for these differences can only be a matter of speculation at this point.

The *ex vivo* expansion of hematopoietic stem and progenitor cells has been described by a number of groups (29–34). Use of IL-3 and IL-6 has previously been reported to enhance the self-renewal and proliferative capacity of primitive progenitors (29) and to improve gene-transfer efficiency (30). IL-3, IL-6, and SCF have also been shown to improve gene transfer into hematopoietic stem cells (31). In our experiments, a period of prestimulation of $CD34^+$ cells by autologous stroma in the presence of cytokines without cell–cell contact was utilized in an effort to enhance transduction efficiency without success. Remaining to be explored is the influence of cell density and purity and of cytokine composition in the medium on transduction efficiency in this system. In contrast to results recently reported in a canine model (35), myeloablation appeared to be necessary in our experiments to allow emergence of genetically modified cells after transplantation. Ultimately, one hopes to achieve sufficient expansion of genetically modified stem cells *ex vivo* to allow efficient repopulation of hematopoietic tissues without myeloablation.

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