H-2d (SF1-1.1; PharMingen, San Diego, CA, USA), which does not react to cynomolgus cells but does react to BALB/c cells.

Teratoma formation

Cynomolgus ES cells (approximately 10^6 cells per site) were injected subcutaneously into the hind leg of 6- to 8-week-old nonobese diabetic/severe combined immunodeficient mice (Jackson Laboratory, Bar Harbor, ME, USA). The resulting tumors (usually 9–12 weeks after the injection) were dissected and fixed in 4% paraformaldehyde. For histological analysis, samples from the tumors were embedded in paraffin and stained with hematoxylin and eosin. To observe GFP fluorescence, samples were embedded in OTC compound (Sakura, Zoeterwoude, Netherlands), frozen, sectioned, and examined under a fluorescence microscope.

Hematopoietic differentiation

The mouse bone marrow stromal cell line OP9 was maintained in α-modified minimum essential medium (Invitrogen) supplemented with 20% FCS as described previously.[28] For induction of hematopoietic differentiation, ES cells were seeded onto a mitomycin C-treated confluent OP9 cell layer in six-well plates. Medium to support the differentiation was described elsewhere.[29] Cells at day 18 were placed in Methocult GF+ media (StemCell Technologies, Vancouver, Canada) at 1×10^4 and 1×10^5 cells per plate and clonogenic hematopoietic colonies were produced. After 14 days, individual colonies were removed and spun onto glass slides. Cells were stained with the Wright–Giemsa method. The nitro blue tetrazolium (NBT, Sigma) reduction test was performed on the cells as a granulocyte functional assay according to a previously described method.[30]

Neural differentiation

The induction of neural differentiation was carried out as described previously.[31] Day-4 embryoid bodies were plated onto tissue culture dishes and nestin-positive cells were selected in DMEM/F12 medium supplemented with 5 μg/ml of insulin (Sigma), 50 μg/ml of transferrin (Sigma), 30 nM selenium chloride (Sigma), and 5 μg/ml of fibronectin (Sigma) for 5 days. Cells were then trypsinized and plated in polyornithine-coated dishes (15 μg/ml) and expanded in N2 medium[32] supplemented with 1 μg/ml of laminin (Sigma) and 10 μg/ml of basic fibroblast growth factor (bFGF; Roche, Basel, Switzerland) for 6 days. Differentiation was induced by removal of bFGF. To confirm the neural differentiation, cells were stained with anti-human MAP-2. Briefly, cells were fixed in 4% paraformaldehyde in PBS and incubated with anti-human MAP-2 (HM-2; Sigma; diluted 1:4000) and then by Alexa Fluor 594-labeled antibody (diluted 1:500; Molecular Probe, Eugene, OR, USA). The samples were examined under a fluorescence microscope.

DNA-PCR

DNA-PCR for the SeV genome and GFP sequences was carried out as follows. DNA was extracted using the QIAamp DNA mini kits (Qiagen, Hilden, Germany) and 250 ng was used for each PCR with ExTaq (Takara, Shiga, Japan). Amplification conditions were 30 cycles of 94°C for 1 min, a variable annealing temperature (noted below) for 1 min, and 72°C for 1 min. The amplified products were run on 2% agarose gel and visualized by ethidium bromide staining. Primer sequences, annealing temperatures and product sizes were as follows: the SeV vector genome sequence: 5′-AGA GAA CAA GAC TAA GGC TAC C-3′ and 5′-ACC TTG ACA ATC CTG ATG TGG-3′ (55°C, 580 bp); the GFP sequence: 5′-CGT CCA GGA GCG CAC CAT CTT C-3′ and 5′-GGT CTT TGC TCA GGG CGG ACT-3′ (60°C, 356 bp). the cynomolgus β-actin sequence: 5′-CAT TGT CAT GGA CTC TGG CGA CGG-3′ and 5′-CAT CTC CTG CTC GAA GTC TAG GGC-3′ (60°C, 234 bp).

RNA-PCR

RNA-PCR for the SeV RNA genomic sequence was carried out as follows. Total RNA was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX, USA). Reverse transcription was conducted by using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). The product (250 ng) after the reverse transcription was used for the subsequent PCR as described above.

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Method — Intra-bone marrow transplantation of hematopoietic stem cells in non-human primates: long-term engraftment without conditioning

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Abstract—It has recently been reported that bone marrow cells can efficiently engraft without marrow conditioning when implanted directly into the bone marrow cavity (intra-bone marrow transplantation, iBMT) in mice. We have successfully examined the efficacy of autologous iBMT in a cynomolgus monkey model in conjuction with an *in vivo* expansion of transplanted cells by a selective amplifier transgene (Ueda *et al.*, 2004) and provide here the detailed parameters of our iBMT method. We injected retrovirally-marked autologous CD34$^+$ cells directly into the non-conditioned marrow cavity of the femur and humerus after gently irrigating the cavity with saline. This transplant procedure was safely performed without pulmonary embolism. Gene-marked cells were not detectable in the peripheral blood at one hour and one day after iBMT as assessed by sensitive PCR, indicating that iBMT hardly generated a systemic delivery of transplanted cells. On the other hand, 2 to 30% of clonogenic hematopoietic colonies produced from the implanted marrow were gene-marked at 6–12 months after iBMT. Our iBMT method for non-human primates is thus discussed in terms of long-lived hematopoietic stem/progenitor cells, bone marrow niche and long-term engraftment after iBMT without myeloablative conditioning.

Keywords: gene marking; hematopoietic stem cell; intra-bone marrow transplantation; non-conditioning; non-human primate.

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INTRODUCTION

One major barrier to effective hematopoietic stem cell (HSC) gene therapy is the necessity for myeloablation before transplantation. Current myeloablative conditioning regimens are associated with high systemic toxicity, and potential damage to marrow stroma possibly resulting in impaired engraftment (Plett *et al.*, 2002). In some disorders such as X-linked severe combined immunodeficiency, however, transplanted genetically-corrected cells increased in number to a therapeutic level without prior conditioning due to a naturally-occurring selective growth advantage (Cavazzana-Calvo *et al.*, 2000). When gene-modified cells do not have such a growth advantage, it is difficult to obtain therapeutic levels of corrected cells *in vivo* after transplantation without prior conditioning (Malech *et al.*, 1997; Dunbar *et al.*, 1998). Conditioning treatment of reduced intensity also results in insufficient engraftment of gene-modified cells in non-human primates (Huhn *et al.*, 1999; Rosenzweig *et al.*, 1999).

It has been reported that bone marrow cells can efficiently engraft without marrow conditioning when implanted directly into the bone marrow cavity in mice (Zhong *et al.*, 2002). Using the iBMT method, human cord blood cells are able to engraft more efficiently in bone marrow of sublethally irradiated immunodeficient mice as compared to the intravenous transplantation method (Mazurier *et al.*, 2003; Yahata *et al.*, 2003; Wang *et al.*, 2003). These observations raise the possibility that transplanted HSCs could engraft after iBMT without marrow conditioning in primates, although iBMT has been shown to be equivalent to intravenous infusion in patients under standard conditioning with cyclophosphamide and either total body iradiation or busulfan (Hagglund *et al.*, 1998). We have successfully examined this possibility in a cynomolgus monkey model in conjuction with an *in vivo* expansion of transplanted cells by a selective amplifier transgene (Ueda *et al.*, 2004) and provide here the detailed parameters of our iBMT method.

MATERIALS AND METHODS

Animals

Cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and the guiding principles for animal experiments using non-human primates formulated by the Primate Society of Japan. The animals were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpes virus B, varicella-zoster-like virus, and measles virus. The protocol of experimental procedures was approved by the animal welfare and animal care committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Collection of CD34$^+$ cells

Peripheral blood or bone marrow cells were collected by leukapheresis or by aspiration from iliac bones, respectively (Ageyama *et al.*, 2002). From harvested cells, a leukocyte fraction was obtained after erythrocyte lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA; Wako, Osaka, Japan). CD34$^+$ cells were concentrated using magnet beads conjugated with anti-human CD34 (clone 561; Dynal, Lake Success, NY, USA) which cross-reacts to cynomolgus CD34 (Shibata *et al.*, 2003). The purity of CD34$^+$ cells ranged from 90 to 95% as assessed with another anti-human CD34 (clone 563; PharMingen, San Diego, CA, USA) which cross-reacts to cynomolgus CD34 (Shibata *et al.*, 2003).

Retroviral transduction

We used the PLI non-expression retroviral vector, which contains non-translated neo^R and β-*gal* sequences (Heim *et al.*, 2000). The titer of viral supernatant was 1×10^6 genomic copies per ml, as assessed by RNA dot blot. CD34$^+$ cells were cultured at a starting concentration of $1–5 \times 10^5$ cells/ml in fresh vector supernatant of PLI with recombinant human (rh) stem cell factor (SCF; Biosource, Camarillo, CA, USA), rh thrombopoietin (Kirin, Tokyo, Japan) and rh Flt-3 ligand (Research Diagnostics, Flanders, NJ, USA) each at 100 ng/ml in dishes coated with 20 μg/cm^2 of RetroNectin (Takara, Shiga, Japan). Every 24 hours, nonadherent cells were collected, spun down, resuspended in fresh vector supernatant and cytokines, and then returned to the same RetroNectin-coated dishes. After 96-hours transduction, cells were washed and the culture was continued (Dulbecco's modified Eagle's medium [Invitrogen, Carlsbad, CA, USA] containing 10% fetal calf serum [Invitrogen]) for two additional days in the same RetroNectin-coated dishes in the presence of rhSCF alone (Takatoku *et al.*, 2001).

Intra-bone marrow transplantation (iBMT)

Cynomolgus monkeys were anesthetized. Two needles were inserted into both ends of the femur and humerus (Kushida *et al.*, 2002) and iBMT was processed as detailed in the Results section. Upon completion of the iBMT, the needle holes were sealed with bone wax (Lukens, Reading, PA, USA).

Measuring intraosseous pressure

A pressure transducer DX-360 (Nihon Koden, Tokyo, Japan) was connected to an intra-femur/humerus needle via a tight-fitting polyethylene tube filled with normal saline (Lucht *et al.*, 1981; Harper *et al.*, 1991). The transducer was calibrated so that the position of the inserted needle tip was a zero level (0 mmHg). Intraosseous pressure was measured and recorded on a polygraph LEG-1000 (Nihon Koden).

Quantitative PCR

Genomic DNA was extracted using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany). DNA (250 ng) was amplified in triplicate with PLI primers (5′-TCC ATC ATG GAT GCA ATG CGG C-3′ and 5′-GAT AGA AGG CGA TGC GCT GCG AAT CG-3′). Standards consisted of DNA extracted from a PLI-producer cell line (which has one copy of the proviral sequence per cell) serially diluted with control cynomolgus genomic DNA. Negative controls consisted of DNA extracted from peripheral blood cells of naive monkeys. A β-actin primer set (5′-CCT ATC AGA AAG TGG TGG CTG G-3′ and 5′-TTG GAC AGC AAG AAA GTG AGC TT-3′) was used to certify equal loading of DNA per reaction. Reactions were run using Qiagen SYBR green PCR master mix (Qiagen) on the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using the following conditions: 50°C for 2 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 62°C for 30 sec, 72°C for 30 sec, and 83°C for 15 sec. The quantitative PCR was certified each time to yield linear amplifications in the range of the intensity of positive control series (0.01–100%, correlation coefficient >0.98). For calculating transduction efficiencies, Ct values of the vector sequence were normalized based on Ct values of the internal control β-actin sequence of the same samples as directed by the manufacturer. Gene marking percentages were calculated given that each vector-positive cell contains one copy of the vector sequence.

Colony PCR

Cells were plated in a 35-mm petri-dish containing 1 ml of α-minimum essential medium (Invitrogen), 1.2% methylcellulose (Shin-Etsu Chemicals, Tokyo, Japan), 100 ng/ml of rh interleukin-3 (PeproTech, Rocky Hill, NJ, USA), 100 ng/ml of rh interleukin-11 (PeproTech), 100 ng/ml of rhSCF (Biosource), 2 U/ml of rh erythropoietin (Roche, Basel, Switzerland), 20% fetal calf serum, 1% bovine serum albumin, 5×10^{-5} M 2-mercaptoetanol (Sigma, St. Louis, MO, USA) and antibiotics (100 U/ml of penicillin and 0.1 mg/ml of streptomycin; Meiji, Tokyo, Japan). Well-separated, individual colonies at day 14 were removed to 50 μl of distilled water, digested with 20 μg/ml of proteinase K (Takara) at 55°C for 1 hour then 99°C for 10 min, and assessed for the vector sequence using PLI primers by nested PCR. The outer primer set was 5′-TCC ATC ATG GAT GCA ATG CGG C-3′ and 5′-GAT AGA AGG CGA TGC GCT GCG AAT CG-3′. The inner primer set was 5′-ATA CGC TTG ATC CGG CTA CCT G-3′ and 5′-GAT ACC GTA AAG CAC GAG GAA G-3′. The outer PCR products were purified using MicroSpin S-400 HR columns (Amersham, Piscataway, NJ, USA) before being subjected to the inner PCR. Amplification conditions for both outer and inner PCR were 20 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min. Simultaneous PCR for the β-actin sequence was also performed to certify amplification of the sample DNA in each colony. The primer set for β-actin PCR was the same as described for the quantitative PCR. Amplification conditions for β-actin PCR were 30 cycles of

95°C for 1 min, 54°C for 1 min, and 72°C for 2 min. PCR products were run on 2% agarose gel. The product sizes were 483 and 232 bp for the PLI vector and β-actin sequences, respectively. Plucked methylcellulose not containing colonies served as negative controls. Transduction efficiency was calculated by dividing the number of colonies positive for the vector sequence by the number positive for the β-actin sequence.

RESULTS

Gene marking of cynomolgus CD34$^+$ cells

Cynomolgus CD34$^+$ cells were collected either from iliac bones or peripheral blood and purified to 90–95% homogeneity. They were genetically marked with a non-expression retroviral vector, *i.e.* aforedescribed PLI which contains non-translated sequences (Heim *et al.*, 2000). The transduction results are summarized in Table 1.

Autologous iBMT

Instead of the conventional intra-venous transplantation (iVT), we injected the gene-marked, autologous CD34$^+$ cells directly into the bone marrow cavity of four proximal limb bones: the femurs and humeri (Fig. 1). Needles were inserted into both ends of the femur or humerus of two anesthetized cynomolgus monkeys (IB3048 and IB3053). A syringe containing 50 ml of heparin-added normal saline was connected to one needle and an empty syringe was connected to the other. The plunger of the empty syringe was pulled to collect marrow, and consequently normal saline in the other syringe was drawn into the marrow cavity (Fig. 1A). Normal saline was thus irrigated gently from one syringe to the other through the marrow cavity without inflicting extra-pressure. This saline irrigation was done twice.

Table 1.
Ex vivo transduction of purified CD34$^+$ cells

Animal no.	CD34$^+$ cell source	Sex, age, body weight	No. of infused CD34$^+$ cells/kg	Transduction efficiency*
IB3048	Bone marrow	Male, 3.1 years, 3.1 kg	4.5×10^7	34/46 (74%)
IB3053	Peripheral blood	Male, 3.2 years, 2.5 kg	8.1×10^6	49/78 (63%)
V0065	Peripheral blood	Male, 5.5 years, 4.4 kg	1.2×10^7	3/45 (6.7%)
V1007	Peripheral blood	Male, 4.1 years, 3.7 kg	1.5×10^6	14/41 (34%)

* As assessed by colony PCR and published in Ueda *et al.*, 2004.

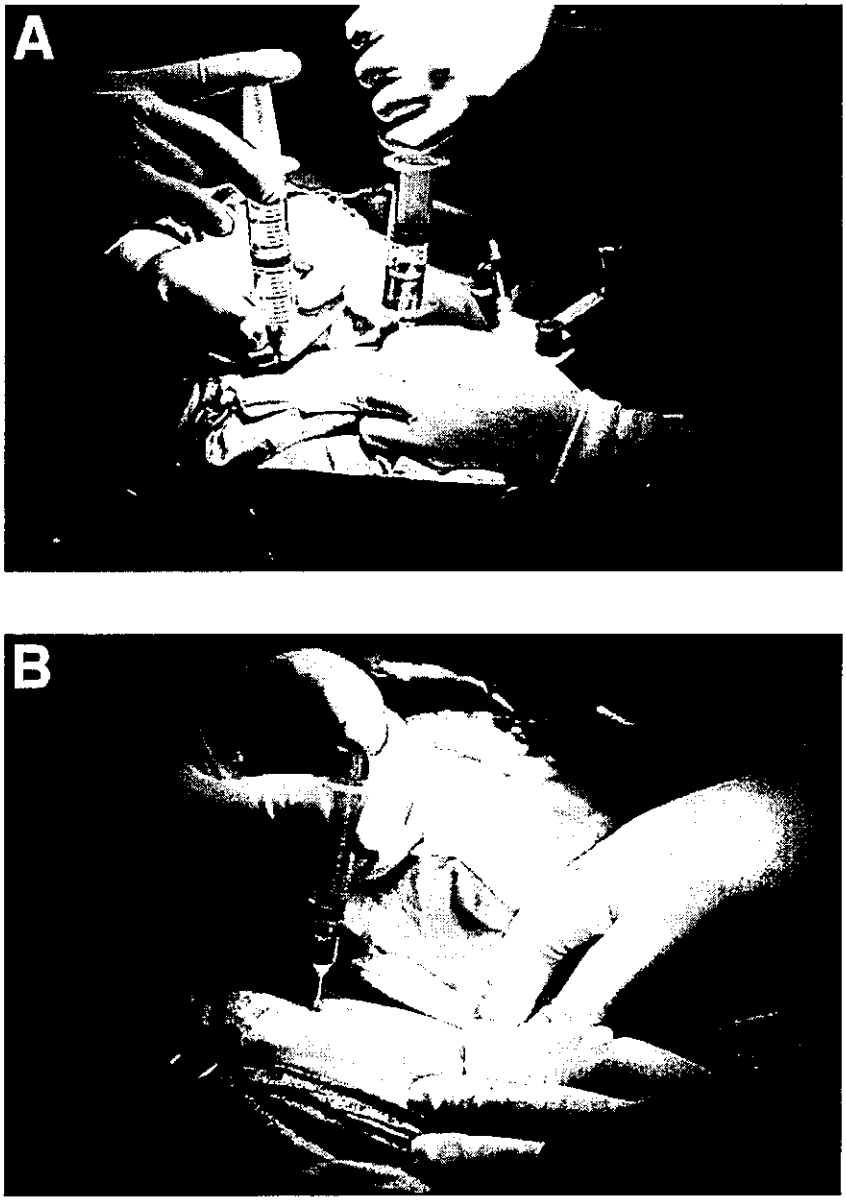

Figure 1. iBMT method. Needles were inserted into either end of the femur and humerus. Normal saline was irrigated gently from one syringe to the other through the marrow cavity (A). Gene-marked cells were then injected into the marrow cavity through one needle (B).

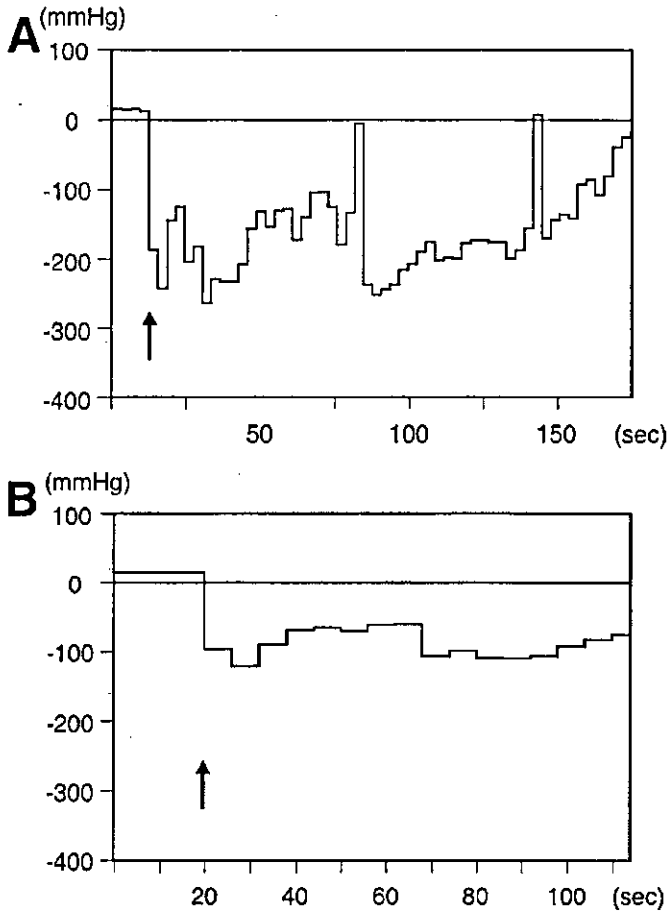

Figure 2. Intraosseous pressure. The internal pressure in the marrow cavity was measured throughout the irrigation of saline in the femur (A) and humerus (B). Representative time-course profiles are shown. Arrows indicate time points of the initiation of saline irrigation. Saline irrigation was conducted without inflicting positive pressure on the marrow cavity.

Gene-marked cells suspended in 1 ml of phosphate-buffered saline containing 10% autologous serum were then injected into the marrow cavity through one needle (Fig. 1B).

In order to rule out the possibility that excessive positive pressure on the marrow cavity during the saline irrigation could cause pulmonary embolism, we tried not to inflict extra-pressure during the procedure. In fact, we have confirmed that saline irrigation was conducted without inflicting positive pressure on the marrow cavity by measuring the intraosseous pressure in one animal (Fig. 2). A third needle was inserted in the middle of the femur or the humerus between the two needles described above, and connected to a pressure transducer. As shown in Fig. 2,

Table 2.
Low-level systemic delivery and long-term engraftment of transplanted cells after iBMT without conditioning

Animal no.	Fractions of gene-marked cells in peripheral blood		Fractions of gene-marked CFU in bone marrow after transplantation	
	One hour after transplantation	One day after transplantation	Implanted femurs	Non-implanted iliac bones*
iBMT				
IB3048	Not detectable (< 0.01%)	Not detectable (< 0.01%)	15/48 (31%) at day 175 1/44 (2.3%) at day 355	3/48 (6.3%) at day 175 1/44 (2.3%) at day 335
IB3053	Not detectable (< 0.01%)	Not detectable (< 0.01%)	1/32 (3.1%) at day 255	4/40 (10%) at day 255
iVT controls				
V0065	0.02%	Not detectable (< 0.01%)	NA#	0/47 at day 125
V1007	0.02%	Not detectable (< 0.01%)	NA	0/4 at day 87

*As published in Ueda *et al.*, 2004.
#NA, non applicable.

the original intraosseous pressure was 10–20 mmHg; after the initiation of saline irrigation, the intraosseous pressure of both femur and humerus stayed below zero mmHg.

The transplant procedure was thus safely performed without pulmonary embolism or other complications. Importantly enough, myeloablative conditioning treatment such as irradiation was not conducted prior to transplantation. The same held true for the two control animals (V0065 and V1007) that were subjected to standard iVT.

Peripheral blood monitoring after iBMT

One hour and one day after iBMT, gene-marked cells were not detectable in the peripheral blood as assessed by quantitative PCR (detectable range > 0.01%; Table 2), indicating that a systemic delivery of transplanted cells hardly occurred following iBMT. On the other hand, gene-marked cells were detectable, albeit at low levels, one hour after control iVT.

However, after a few days, very low but significant levels (0.01–0.1%) of gene-marked circulating cells were clearly monitored (Ueda *et al.*, 2004). Notably enough, unlike iVT controls, iBMT IB3048 monkey maintained a detectable level of circulating marked cells until the end of the experiment, *i.e.* a whole year (Ueda *et al.*, 2004).

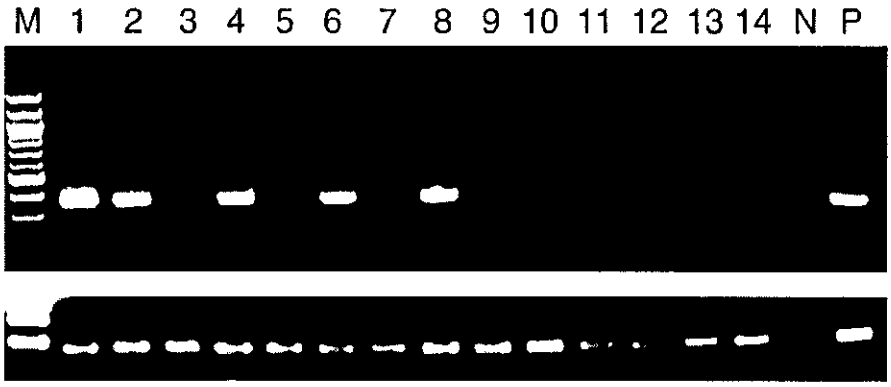

Figure 3. Engraftment of transplanted cells after iBMT without prior conditioning. Cells taken from the implanted femoral bone marrow were subjected to a clonogenic progenitor assay at 6–12 months after iBMT. Individual colonies were examined for the vector sequence (upper panel) and internal control β-actin sequence (lower panel) by PCR. In representative results shown here, five colonies (no. 1, 2, 4, 6, and 8) were vector-positive among 14 of β-actin-positive colonies. M, molecular weight marker; lanes 1–14, individual colony numbers; N, negative control (methylcellulose medium alone); P, positive control.

Long-term engraftment of transplanted cells after iBMT

Cells taken from the implanted femoral bone marrow were subjected to a clonogenic progenitor assay at 6 to 12 months after iBMT. The resulting colonies (colony-forming units, CFU) were examined for the vector sequence by PCR (Fig. 3). As summarized in Table 2, 2 to 30% of CFU were found gene-marked. This result suggests that transplanted cells can engraft in non-conditioned recipient monkeys after iBMT.

Importantly enough, gene-marked CFUs were also found in nonimplanted iliac marrow (Table 2). Indeed, we have shown that such a translocation of marked progenitor cells to nonimplanted bone marrow was detectable within two weeks after iBMT (Ueda *et al.*, 2004). In contrast to iBMT, control iVT is not associated with long-term detection of marked CFUs in iliac marrow as clearly shown in the monkey V0065 (Table 2 and Ueda *et al.*, 2004).

DISCUSSION

In this article, we have detailed our iBMT method for long-term engraftment of autologous $CD34^+$ cells without prior myeloablative conditioning in a primate model. We showed here and in another article (Ueda *et al.*, 2004) that autologous $CD34^+$ cells transplanted directly into both femoral and humeral bone marrow engrafted without prior conditioning in cynomolgus monkeys. Such an engraftment extended until the end of the experiment, *i.e.* lasted 12 months and over 8 months respectively in the two monkeys that have been used in this experiment. Importantly

enough, we did not find any gene-marked CFU (0/47) at 4 months post-transplant in the relevant monkey control in which marked autologous $CD34^+$ cells were intravenously infused under non-myeloablative conditions. Although gained with statistically non-significant numbers of cynomolgus monkeys and low level marking of iVT cells (Table 1), our data suggest that, under non-myeloablative conditions, iBMT is more effective than conventional iVT in our primate model.

There are several possible explanations for the relatively efficient engraftment after iBMT as compared to iVT. The iBMT method may allow many more HSCs and progenitor cells to reach the bone marrow without being trapped at other sites in the body such as the liver, spleen, and lung, than the iVT method. Directly injected cells may also be able to find a proper bone marrow niche for engraftment relatively easily as compared to intravenously injected cells. In fact, it has been reported that some fraction of HSCs are unable to reach the bone marrow niche via conventional venous route (Wang *et al.*, 2003; Mazurier *et al.*, 2003). In the current study, toxic conditioning such as irradiation therapy was not conducted. Thus, there was no conditioning-related injury of stroma cells. This might increase engraftment, given that the injury of stroma cells may result in impaired engraftment (Plett *et al.*, 2002). In addition, it is possible that the physical elimination of endogenous marrow with saline before injection increased engraftment.

Interestingly, although we showed that transplanted cells did not detectably enter the systemic flow (Table 2), gene-marked CFU were also found in the non-implanted iliac marrow at comparable levels after iBMT (Table 2, and Ueda *et al.*, 2004). Thus, transplanted cells relocated from an implanted bone to another, although iBMT in itself did not create a systemic distribution of transplanted cells. A similar translocation post-transplantation has also been reported in mouse syngeneic and allogeneic iBMT and human-mouse xeno-iBMT models (Askenasy *et al.*, 2002; Zhong *et al.*, 2002; Mazurier *et al.*, 2003; Yahata *et al.*, 2003; Wang *et al.*, 2003).

Importantly enough, the very low level of gene-marked cells that has been detected long-term after a few days post-iBMT in peripheral blood of the monkey IB3048 (Results section and Ueda *et al.*, 2004) is consistent with the long-term engraftment of active HSCs. Like for bone marrow CFUs, gene-marking of circulating cells has been maintained until the end of the experiment, *i.e.* a whole year. Such a low level of gene-marked circulating cells is not enough for a therapeutic trial in which a non-conditioning regimen would be required such as for the gene therapy of chronic granulomatous disease and Gaucher disease. However, when the iBMT method is combined with an *in vivo* selection method such as the use of a selective amplifier gene, engraftment of transduced cells at low levels may allow successful expansion to clinically relevant levels even without marrow conditioning (Ueda *et al.*, 2004). Furthermore, this iBMT method may be applicable to allogeneic HSC transplantation (Askenasy *et al.*, 2002; Esumi *et al.*, 2003; Nakamura *et al.*, 2004). It might also be effective for mesenchymal stem cell transplantation, given that these cells express low levels of homing receptors

(Wynn *et al.*, 2004): iBMT would allow such cells to engraft in bone marrow more efficiently than iVT.

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High-Level *in Vivo* Gene Marking after Gene-Modified Autologous Hematopoietic Stem Cell Transplantation without Marrow Conditioning in Nonhuman Primates

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The successful engraftment of genetically modified hematopoietic stem cells (HSCs) without toxic conditioning is a desired goal for HSC gene therapy. To this end, we have examined the combination of intrabone marrow transplantation (iBMT) and *in vivo* expansion by a selective amplifier gene (SAG) in a nonhuman primate model. The SAG is a chimeric gene consisting of the erythropoietin (EPO) receptor gene (as a molecular switch) and c-Mpl gene (as a signal generator). Cynomolgus CD34$^+$ cells were retrovirally transduced with or without SAG and returned into the femur and humerus following irrigation with saline without prior conditioning. After iBMT without SAG, 2–30% of colony-forming cells were gene marked over 1 year. The marking levels in the peripheral blood, however, remained low (<0.1%). These results indicate that transplanted cells can engraft without conditioning after iBMT, but *in vivo* expansion is limited. On the other hand, after iBMT with SAG, the peripheral marking levels increased more than 20-fold (up to 8–9%) in response to EPO even at 1 year posttransplant. The increase was EPO-dependent, multilineage, polyclonal, and repeatable. Our results suggest that the combination of iBMT and SAG allows efficient *in vivo* gene transduction without marrow conditioning.

Key Words: gene therapy, hematopoietic stem cell, intrabone marrow transplantation, nonconditioning, *in vivo* expansion, selective amplifier gene, nonhuman primate

Introduction

The ability to expand selectively cells containing potentially therapeutic genes *in vivo* would represent an important tool for the clinical application of hematopoietic stem cell (HSC)-based gene transfer. This would circumvent low gene transfer efficiency into HSCs, which is one of the current limitations of this promising technology. Furthermore, the ability to expand genetically modified cells *in vivo* would circumvent another major problem of HSC gene therapy; myeloablative conditioning is necessary unless gene-modified cells have clear growth advantage [1]. Current myeloablative conditioning regimens are associated with high systemic toxicity and potential damage to marrow stroma, possibly resulting in impaired engraftment [2]. With the *in vivo* selection method using a drug-resistance gene, engraftment of transduced cells at low levels may allow successful expansion to clinically relevant levels even without marrow conditioning, although the administration of cytotoxic agents is required for the selection [3]. It has recently been reported that bone marrow cells can efficiently engraft mice without marrow conditioning when implanted directly into the

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bone marrow cavity (intrabone marrow transplantation, iBMT) [4,5]. Using the iBMT method, human cord blood cells are also able to engraft efficiently in bone marrow of sublethally irradiated immunodeficient mice [6–8]. Although the iBMT method has been successful in mice, the efficacy in primates remains to be examined.

We have previously developed a selective amplifier gene (SAG) consisting of a chimeric gene encoding the granulocyte colony-stimulating factor (G-CSF) receptor (as a growth-signal generator) and the hormone-binding domain of the steroid receptor (as a molecular switch) [9]. Hematopoietic cells genetically engineered to express this SAG can be expanded in a steroid-dependent manner *in vitro* and *in vivo* in mice and nonhuman primates [10,11]. Here we have examined such expansion in the setting of nonhuman primate iBMT without marrow conditioning using a new SAG encoding the erythropoietin (EPO) receptor (as a molecular switch) and thrombopoietin receptor (c-Mpl; as a signal generator) [12].

RESULTS

Engraftment after iBMT

First, we examined whether gene-marked CD34$^+$ cells engraft after iBMT using two cynomolgus macaques. Cynomolgus CD34$^+$ cells were transduced with the nonexpression retroviral vector PLI (which contains untranslated sequence) [13]. The transduction results are summarized in Table 1. We injected the transduced CD34$^+$ cells directly into the bone marrow cavity of four proximal limb bones (the femurs and humeri) after gently irrigating the cavity with saline (Fig. 1). This transplant procedure was safely performed without pulmonary embolism or infection of bone marrow. Conditioning treatment such as irradiation was not conducted prior to transplantation. In addition, we returned the transduced CD34$^+$ cells into two monkeys by the conventional transplantation method without prior conditioning.

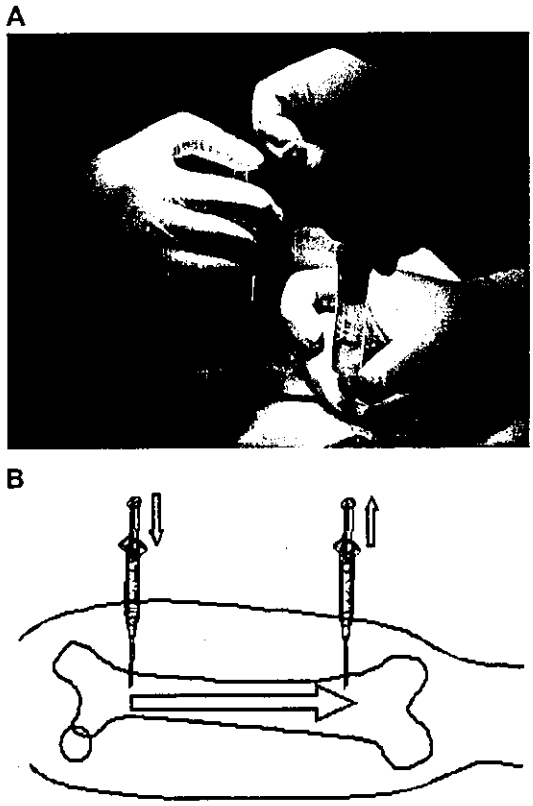

FIG. 1. The iBMT method. We inserted needles at both ends of limb bones (femurs and humeri) and irrigated the bone marrow cavity gently with saline without inflicting extra pressure (A, photo; B, schematic diagram). Gene-modified CD34$^+$ cells were then injected directly into the bone marrow through the needle on one side.

After iBMT, we plated cells from the nonimplanted iliac marrow in methylcellulose medium. We examined the resulting colonies (colony-forming units, CFU) for the provirus by PCR (Fig. 2A and 2B). Two to 30% of colonies (overall 14.2% (74/522)) were positive for the

TABLE 1: *Ex vivo* transduction

Animal	Target cell source	Vector	No. of infused CD34$^+$ cells/kg	Fraction of provirus-positive CFUs in infused CD34$^+$ cells
		Intrabone marrow transplantation		
IB3048	Bone marrow	PLI	4.5×10^7	34/46 (73.9%)
IB3053	Peripheral blood	PLI	8.1×10^6	49/78 (62.8%)
S9042	Peripheral blood	SAG	2.6×10^7	20/35 (57.1%)
S3047	Peripheral blood	SAG	8.1×10^6	11/21 (52.4%)
D8058	Peripheral blood	SAG	7.8×10^5	11/43 (25.6%)
		PLI	5.7×10^5	9/42 (21.4%)
		Intravenous transplantation		
V0065	Peripheral blood	PLI	1.2×10^7	3/45 (6.7%)
V1007	Peripheral blood	PLI	1.5×10^6	14/41 (34.1%)

PLI, nonexpression vector; SAG, selective amplifier gene vector.

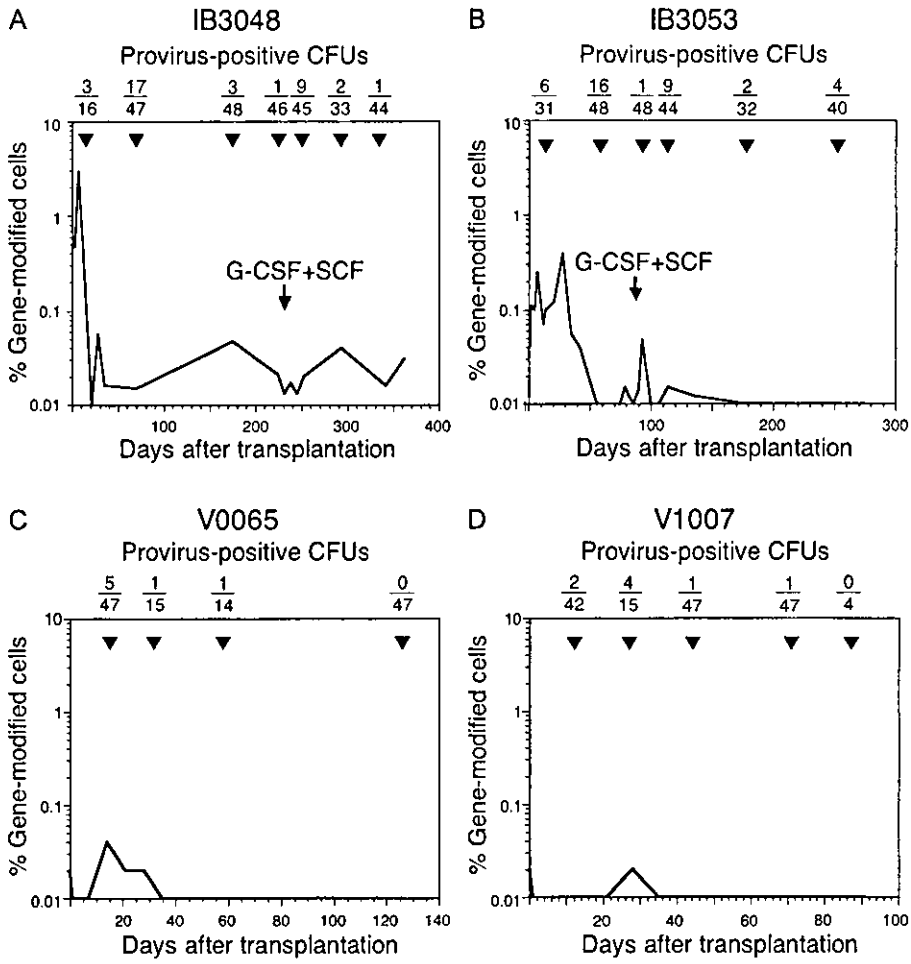

FIG. 2. *In vivo* marking after iBMT and intravenous transplantation without marrow conditioning. CD34$^+$ cells were transduced with nonexpression retroviral vector PLI and returned by iBMT (A, IB3048, and B, IB3053) or by intravenous transplantation (C, V0065, and D, V1007) without conditioning. The upper row shows ratios of provirus-positive CFUs to β-actin-positive CFUs taken from the nonimplanted marrow at time points indicated by arrows. Overall number of provirus-positive CFUs versus overall number of β-actin-positive CFUs was 74/522 (14.2%) for iBMT (A and B) and 15/274 (5.5%) for the intravenous transplantation (C and D). The lower diagram shows percentages of gene-modified cells in the peripheral blood as assessed by quantitative PCR.

provirus and this high marking level persisted for over 1 year posttransplantation. On the other hand, after the conventional intravenous transplantation, generally fewer CFU contained the provirus (overall 5.5% (15/274)) in the bone marrow (Fig. 2C and 2D). Interestingly, the provirus in CFU from the nonimplanted marrow was detectable within 2 weeks after iBMT. Thus, transplanted cells relocated from an implanted bone to another at early time points. A similarly early translocation posttransplantation has also been reported in mouse syngeneic iBMT and human-mouse xeno-iBMT models [4,6–8]. We also examined peripheral blood cells for the provirus by quantitative PCR (Fig. 2A and 2B). The marking levels were, however, very low (<0.1%) in the peripheral blood.

Taken together, these results suggest that transplanted cells can engraft nonconditioned recipients after iBMT but their contribution to the peripheral blood is minimal compared to myeloablated recipients. The cells stay at a resting state in bone marrow without proliferation. In an attempt to proliferate and mobilize iBMT-engrafted resting progenitor cells, we administered G-CSF and stem cell factor (SCF) for 5 consecutive days [14]; however, no obvious increase in the vector-containing cells was observed in the peripheral blood (Fig. 2A and 2B).

EPO-dependent expansion with SAG

We constructed a retroviral vector expressing an SAG that is a chimeric gene of the human EPO receptor gene (extra- plus transmembrane region as a molecular switch) and the human c-Mpl gene (cytoplasmic region as a signal generator) [12]. Cells genetically engineered to express this SAG will proliferate in an EPO-dependent manner. We transduced cynomolgus CD34$^+$ cells with the SAG retroviral vector and introduced them into nonconditioned autologous recipients by iBMT (Table 1). *In vivo* results after transplantation are summarized in Table 2.

In one animal (Fig. 3A), EPO administration triggered a striking elevation in marking levels (7.4% at day 105 posttransplantation) in the peripheral blood. The level of marking in the periphery stayed high for the duration of EPO administration. After cessation of EPO, the level fell to <0.1%. Resumption of EPO administration produced a

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TABLE 2: *In vivo* expansion with SAG after iBMT

	EPO treatment			Marked leukocytes (%)[a]	
Animal	Treatment course	Period (days posttransplant)	Dosage	Basal marking before treatment	Peak marking after treatment (day posttransplant)
S9042	1	1–40	200 IU/kg once daily	NA	7.36% (day 105)
		41–100	200 IU/kg twice daily	NA	7.36% (day 105)
	2	132–210	200 IU/kg twice daily	0.02%	7.72% (day 188)
	3	246–367	200 IU/kg twice daily	0.41%	8.90% (day 348)
S3047	1	75–134	200 IU/kg once daily	0.01%	0.23% (day 145)
		135–166	200 IU/kg twice daily	0.01%	0.23% (day 145)
	2	210–289	200 IU/kg twice daily	0.02%	0.00% (day 289)
D8058	1	1–86	200 IU/kg twice daily	NA	2.30% (day 14)

[a] As assessed by quantitative PCR (see Materials and Methods). NA, not applicable.

similar elevation in the marking levels. The third EPO administration again resulted in the increased marking levels to 8.9% at day 348 posttransplantation. EPO administration was associated with a mild increase in hematocrit (up to 63.5%), which was manageable by occasional phlebotomy. No other adverse effects were observed.

In another animal (Fig. 3B), the SAG-transduced cells increased following transplantation even without exogenous EPO administration. The increase may have been due to increased endogenous EPO elevation resulting from anemia present in the second animal. Overall marking fell with resolution of the anemia. Following resolution, EPO was administered, resulting in an increase in marking levels by more than 20-fold. Marking levels declined to the basal level after discontinuation of EPO. A second attempt to increase marking levels failed, with clearance of SAG-positive cells from the periphery within a month after the second administration, most likely due to cellular immune responses to the xenogeneic SAG (see below).

Multilineage and Polyclonal Expansion

In situ PCR for the proviral sequence showed many transduced cells in the peripheral blood taken from animal S9042 receiving EPO at day 89 posttransplantation (Fig. 4A). We subjected granulocytes and T and B lymphocytes sorted from the peripheral blood of this animal at day 91 posttransplantation to semiquantitative PCR for the provirus. The provirus-containing fraction in granulocytes was 6% and that in B and T lymphocytes was 2% (Fig. 4B), thus indicating that multilineage expansion had occurred. The persistence of marked, short-lived granulocytes for the long term is also another evidence of the successful engraftment of gene-modified HSCs after iBMT. The integration site analysis using the linear amplification-mediated (LAM) PCR method [15]

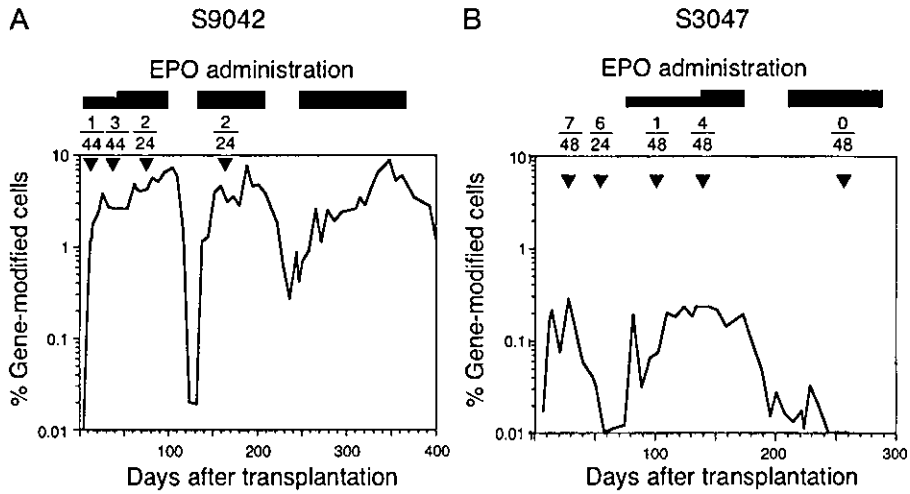

FIG. 3. Expansion of SAG-transduced cells by treatment with EPO after iBMT. $CD34^+$ cells transduced with SAG were returned to each animal by iBMT without conditioning. The animals (A) S9042 and (B) S3047 received EPO at 200 IU/kg once or twice daily (indicated by closed bars). The upper row shows ratios of provirus-positive CFUs to β-actin-positive CFUs taken from the nonimplanted marrow at time points indicated by arrows. The lower diagram shows percentages of gene-modified cells in the peripheral blood as assessed by quantitative PCR.

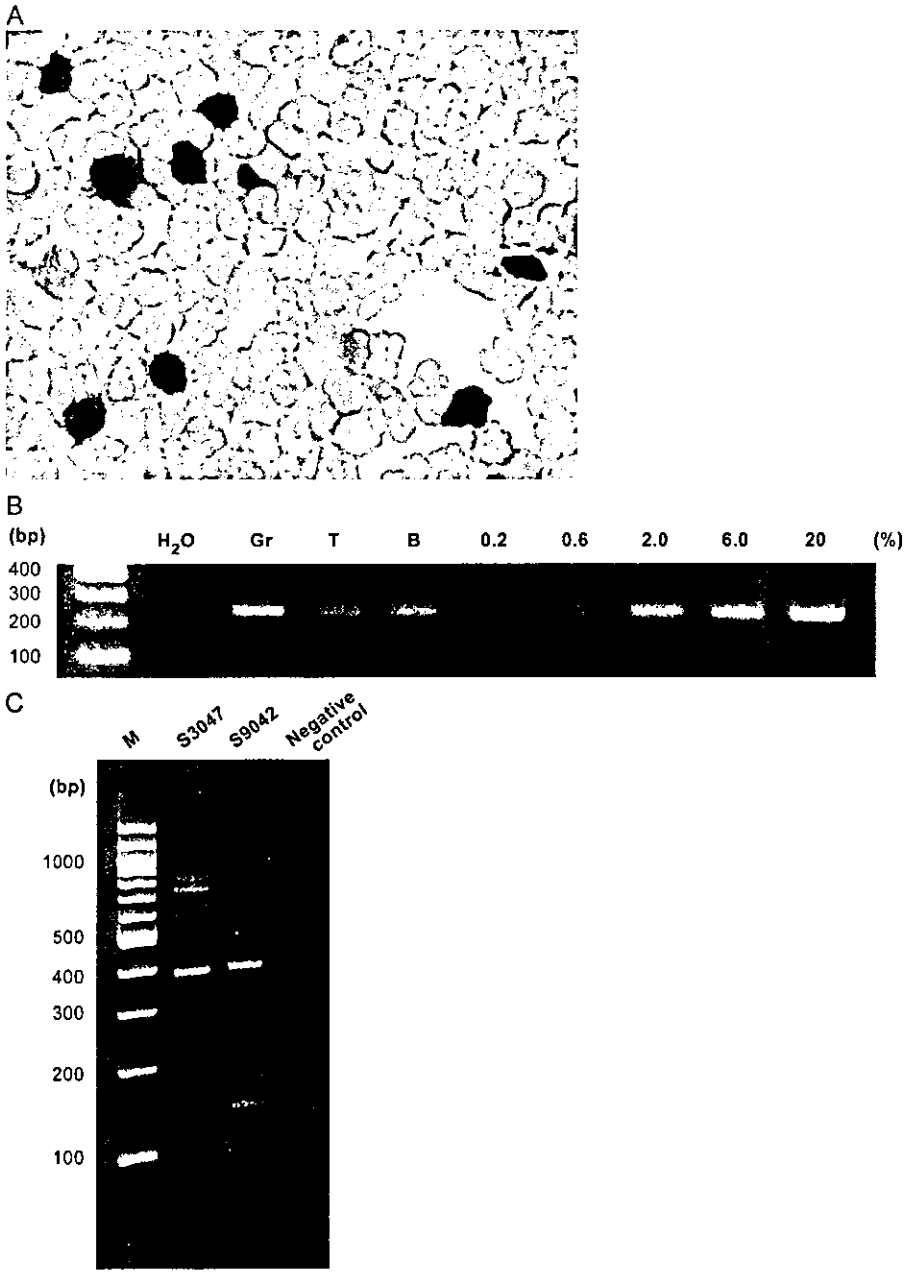

FIG. 4. High-level, multilineage, and polyclonal expansion of gene-modified cells in the peripheral blood after iBMT with SAG in nonconditioned recipients. (A) *In situ* PCR for the provirus. Peripheral blood nucleated cells were collected from animal S9042 receiving EPO at day 89 posttransplantation. Many SAG-transduced cells (stained in black) were detected by *in situ* PCR. (B) Lineage analysis by semiquantitative PCR. DNA from granulocytes (Gr) and T and B lymphocytes sorted from animal S9042 receiving EPO at day 91 posttransplantation was examined for the provirus by semiquantitative PCR. Positive controls corresponding to 0.2, 0.6, 2.0, 6.0, and 20% of transduced cells in peripheral blood were included. (C) Clonal analysis by LAM-PCR. Genomic DNA from peripheral blood of the animals receiving EPO (S9042 at day 90 and S3047 at day 150 posttransplantation) was analyzed by LAM-PCR. Each band indicates different integrants. Negative control was genomic DNA from a naive monkey. M, molecular weight marker.

indicates that the expansion of transduced cells in response to EPO was polyclonal, not mono- or oligoclonal (Fig. 4C).

Dual-Marking Study

We then compared the effects of the SAG vector to a non-SAG vector within, rather than between, individual animals. We harvested cytokine-mobilized peripheral blood $CD34^+$ cells and split them into two equal aliquots. We transduced one aliquot with the SAG vector and the other with the control nonexpression vector (PLI). We mixed both aliquots and returned them by iBMT without marrow conditioning. The animal received EPO from the day after transplantation, and we examined *in vivo* marking levels derived from the two populations by quantitative PCR.

Cells containing the SAG vector increased by 2 logs in the peripheral blood in response to EPO, although cells containing the nonexpression vector remained at low levels (Fig. 5). However, SAG-containing cells were rapidly cleared within 1 month posttransplantation from the periphery and overall SAG-vector marking

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levels became even lower than those from the nonexpression vector-marked fraction. Since cyclosporin A was concomitantly administered to prevent immune responses to human EPO, human EPO concentrations were maintained within an effective range. Thus, it is unlikely that the clearance of xenogeneic human EPO due to immune responses turned off the molecular switch of SAG, resulting in the decrease in SAG-transduced cells.

Immune Responses

The current SAG is a chimeric gene of human origin (the human EPO receptor and human c-Mpl). We collected peripheral lymphocytes from the animal receiving both SAG and nonexpressing PLI (D8058, Fig. 5) at day 169 posttransplantation and examined whether the lymphocytes responded to the xenogeneic SAG *in vitro* (Fig. 6). The response to SAG-transduced target cells was stronger than that to nontransduced target cells ($P = 0.05$), while the response to PLI-transduced target cells did not differ significantly from that to nontransduced target cells ($P = 0.13$). The cellular immune response is, therefore, the most likely reason for the clearance of SAG-transduced cells in this animal. This is not novel, but it has been reported that immune responses against transgene products recognized as foreign can indeed be a major obstacle to long-term persistence of gene-modified cells *in vivo* [13,16,17]. In the human clinical setting, however, immune responses should not occur against SAG, because the SAG is made of human genes.

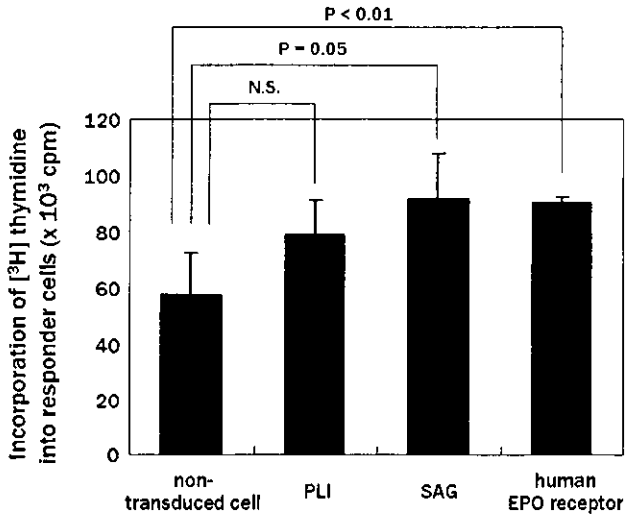

FIG. 6. Positive blastogenic response of lymphocytes to SAG. Peripheral blood mononuclear cells (responder cells) were isolated from monkey D8058 at day 169 posttransplantation (Fig. 5) and cocultured with stimulator cells. The stimulator cells were autologous stromal cells untransduced or transduced retrovirally with PLI, SAG, or human EPO receptor cDNA followed by irradiation with 4000 cGy. After 5 days in culture, the blastogenesis of responder cells was assessed by counting the [^{3}H]thymidine incorporation into responder cells. The averages ± SD of triplicate experiments are shown. N.S., not significant.

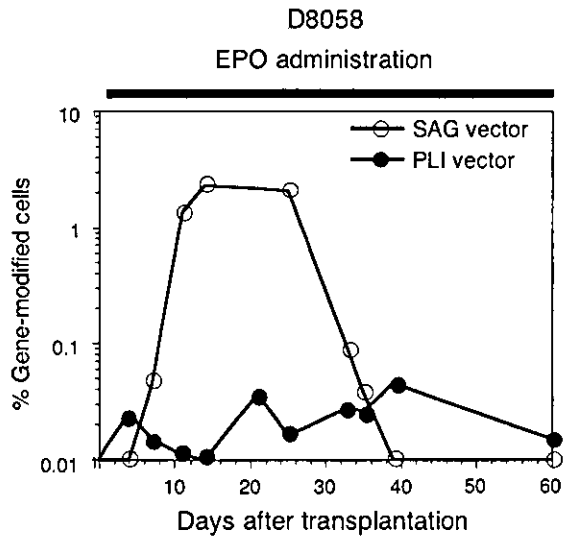

FIG. 5. Dual genetic marking study. CD34$^+$ cells from monkey D8058 were split into two equal aliquots; one aliquot was transduced with SAG vector and the other with nonexpression PLI vector. Both aliquots were together returned to the bone marrow cavity by iBMT without conditioning. EPO (200 IU/kg, twice daily) was administered from the day after transplantation (indicated by a closed bar).

Discussion

Previous papers documented that, without marrow conditioning, very low levels (much less than 0.1%) of cells were marked (or corrected) after CD34$^+$ cell gene therapy of chronic granulomatous disease and Gaucher disease [18,19]. This clinical observation has formed the foundation for the contention that myeloablation (or at least conditioning of reduced intensity) is necessary for successful engraftment of transplanted, genetically modified cells. Our results, however, suggest that nonconditioned iBMT results in much higher gene marking levels (up to 8–9%) through the utilization of an SAG. The physical elimination of endogenous marrow with saline before injection might increase gene marking. In the current study, the marrow of four proximal limb bones (femurs and humeri) was replaced with transplanted cells. If other bones such as the iliac bone (which contains more marrow) are similarly used for iBMT, even higher *in vivo* marking levels may be achieved using an SAG.

Expansion of SAG-transduced cells was seen in three lineages: granulocytes, B lymphocytes, and T lymphocytes. The c-Mpl signal generated by the SAG may work even in lymphocytes. In fact, B lymphocytes were shown to be increased by the activated c-Mpl in a canine trans-