HUMAN GENE THERAPY 15:251–262 (March 2004)

Anti-Human Immunodeficiency Virus Hematopoietic Progenitor Cell-Delivered Ribozyme in a Phase I Study: Myeloid and Lymphoid Reconstitution in Human Immunodeficiency Virus Type-1–Infected Patients

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ABSTRACT

A phase I gene transfer clinical study was undertaken to examine the ability to introduce a potential anti-human immunodeficiency virus (HIV) gene therapeutic into hematopoietic progenitor cells (HPC), thereby contributing to multilineage engraftment. The potential therapeutic effect of genetically modifying HPC with protective genes in HIV-infected adults depends in part on the presence of adult thymic activity and myeloid capacity in the setting of HIV replication. Herein we report the presence and expression of a retroviral vector encoding an anti-HIV-1 ribozyme in mature hematopoietic cells of different lineages, and *de novo* T-lymphocyte development ensuing from genetically engineered CD34$^+$ HPC. Sustained output of vector-containing mature myeloid and T-lymphoid cells was detected even in patients with multidrug-resistant infection. In addition, the study showed that the degree of persistence of gene-containing cells was dependent on transduced HPC dose. These novel findings support the concept of gene therapy as a modality to effect immune reconstitution with cells engineered to inhibit HIV replication and this report represents the first demonstration of long-term maintenance of a potential therapeutic transgene in HIV disease.

OVERVIEW SUMMARY

An anti-human immunodeficiency virus (HIV) ribozyme targeted to a highly conserved region of the HIV-1 genome present in both the *tat* and *vpr* genes was introduced into HIV-positive patients to analyze the feasibility of this approach. Ribozyme and control vector containing hematopoietic cells could be detected in all patients and sustained multilineage engraftment was seen in the patients who received the greatest number of ribozyme-containing CD34$^+$ hematopoietic progenitor cells. In addition, expression of the constructs could be detected in isolated peripheral blood mononuclear cells without the need for *ex vivo* stimulation. The lineages containing gene-marked cells included peripheral blood mononuclear cells, CD4$^+$ and CD8$^+$ T lymphocytes, monocytes, and granulocytes. In addition, naïve CD4$^+$ and CD8$^+$ T lymphocytes showed gene-marking. Taken together these data indicate *de novo* production of myeloid and lymphoid cells from the introduced gene-containing CD34 hematopoietic progenitor cells (HPC).

INTRODUCTION

WE HAVE PREVIOUSLY REPORTED the protocol for a phase I gene transfer study (Amado *et al.*, 1999). The concept

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that this work sought to explore was whether a paradigm for gene therapy for human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) could involve the use of hematopoietic progenitor cells (HPC) as transduction targets thereby replacing a fraction of the HIV-infected cellular pool with cells engineered to inhibit virus replication. This strategy can potentially contribute to virus eradication by protecting $CD4^+$ cells and by allowing the establishment of an antiviral response mediated by protected immune elements. Although studies have demonstrated that the adult uninfected thymus maintains the ability to support T-lymphopoiesis (Jamieson *et al.*, 1999; Poulin *et al.*, 1999), it has not yet been proven that HPC gene therapy can result in the prolonged restoration of the immune system with cells engineered to inhibit HIV-1 replication. While the emergence of recent thymic emigrants in the periphery has been described for patients who were previously antiretroviral therapy (ART)-naive, it was sustained only as long as viremia was kept in check (Douek *et al.*, 1998; Zhang *et al.*, 1999). It is not known whether a similar response would occur in patients with more advanced HIV infection in the context of drug resistance and uncontrolled viremia. In addition, the source of progenitors that gives rise to these recent thymic emigrants has not been elucidated; it is not known whether hematopoietic precursors responsible for the degree of thymopoiesis observed after ART in adults migrate from the bone marrow to the thymus as a response to T-cell depletion, or whether T-lymphoid development after ART derives from T-lymphoid progenitors that colonized the thymus earlier in life. It should also be considered that potential factors inherent to the methods used in genetic manipulation of hematopoietic progenitors might affect their ability to undergo T-lymphoid development. These previously identified factors include the induction of progenitors into cell cycle in preparation for transduction with murine retroviruses (Roe *et al.*, 1993), which could result in myeloid lineage commitment, and the presence of a constitutively expressed foreign gene that might interfere with the required processes of progenitor cell migration, homing and differentiation. By genetically modifying hematopoietic progenitors, our study sought to determine whether myeloid and T-lymphocytes (specifically naïve T lymphocytes) could arise in the setting of HIV replication, and whether myeloid and T-cell development can persist long term in infected adult patients in the presence of detectable viremia.

Ribozymes are RNA structures that possess catalytic activity (Haseloff and Gerlach, 1988). Rz2, the ribozyme used for these studies, is a hammerhead ribozyme directed against the translational initiation region of the HIV-1 *tat* gene (Sun *et al.*, 1995b). The target sequence of Rz2 is at position 5833 to 5849 within the HIV-1 strain HXB2 (GGAGCCAGTA_GATCCTA; Genbank sequence K03455), where cleavage occurs after the GUA triplet at position 5842. We have previously shown that the stable introduction of this ribozyme into T-lymphocyte lines and peripheral blood lymphocytes (PBL) results in significant inhibition of HIV-1 replication (Sun *et al.*, 1994, 1995a). Rz2 has also been shown to confer protection against HIV-1 without the development of resistance in a multiple passage assay (Wang *et al.*, 1998).

We performed a phase I gene transfer clinical study to investigate whether (1) the introduction of an anti-HIV-1 ribozyme into HPC could result in the emergence of thymic emigrants bearing vector sequences, (2) normal T-lymphocyte and myeloid maturation could take place in genetically modified cells, and (3) vector presence and expression could persist long-term. In addition, assessment was made of any preferential survival of ribozyme-containing cells (Amado *et al.*, 1999).

MATERIALS AND METHODS

Study design

For retroviral transduction, Rz2 was cloned into a *Sal*I site of the untranslated region of the neomycin phosphotransferase (neo^R) gene of the Moloney murine leukemia virus (MoMLV) retroviral vector LNL6 (Bender *et al.*, 1987) where it is expressed as a *neo*-ribozyme transcript from the MoMLV long terminal repeat (LTR). The resulting vector was termed RRz2 and its structure as well as the Rz2 target site and sequence are shown in Figure 1. To control for potential ribozyme-specific effects on progenitor cell engraftment and T-lymphoid development, and to study potential effects on T-lymphocyte survival conferred by Rz2, progenitor cells were also transduced with the control retroviral vector LNL6. Study details are as previously described (Amado *et al.*, 1999). The study, which was approved by the National Institutes of Health Recombinant DNA Advisory Committee (NIH RAC), the Food and Drug Administration (FDA), and the UCLA Institutional Review Board, was conducted in accordance with the tenets of the Helsinski protocol. Briefly, patients with viremia less than 10,000 copies per milliliter and CD4 counts between 300 and 700 cells/mm^3 underwent mobilization of peripheral blood progenitor cells (PBPC) with the cytokine granulocyte-colony stimulating factor (G-CSF) at a dose of 10 μg/kg daily for 6 days. PBPC procurement was carried out via apheresis on days 5 and 6 of G-CSF treatment using the COBE® Spectra™ Apheresis System (Gambro BCT, Lakewood, CO). $CD34^+$ cell selection was performed using the CEPRATE® SC Stem Cell Concentration System (CellPro Inc., Bothell, WA) (patients 1–7) and Isolex 300i cell selection system (Nexell Therapeutics, Irvine, CA) (patients 8–10). After purification of PBPC for CD34 surface marker expression, cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) and induced into cycle using the cytokine combination of megakaryocyte growth and development factor (MGDF) and stem cell factor (SCF) at a concentration of 100 ng/ml and 50 ng/ml, respectively (Amgen Inc., Thousand Oaks, CA; Amado *et al.*, 1998). Approximately equal numbers of $CD34^+$ cells were transduced independently with the RRz2 and LNL6 vectors. After transduction, cells were pooled and after appropriate safety checks, infused into autologous recipient patients without myelosuppression.

Vector production and retroviral transduction of $CD34^+$ cells

The LNL6 and RRz2 producer cell lines were prepared in a two-stage process by transfecting the cDNA constructs, pLNL6 or pRRz2, into the psi2 packaging cell line to produce two populations of ecotropic replication-incompetent virus. These two populations were then used to infect the PA317 amphotropic packaging cell line (Miller and Buttimore, 1986). Clonal producer cell lines derived after selection in G418 were checked

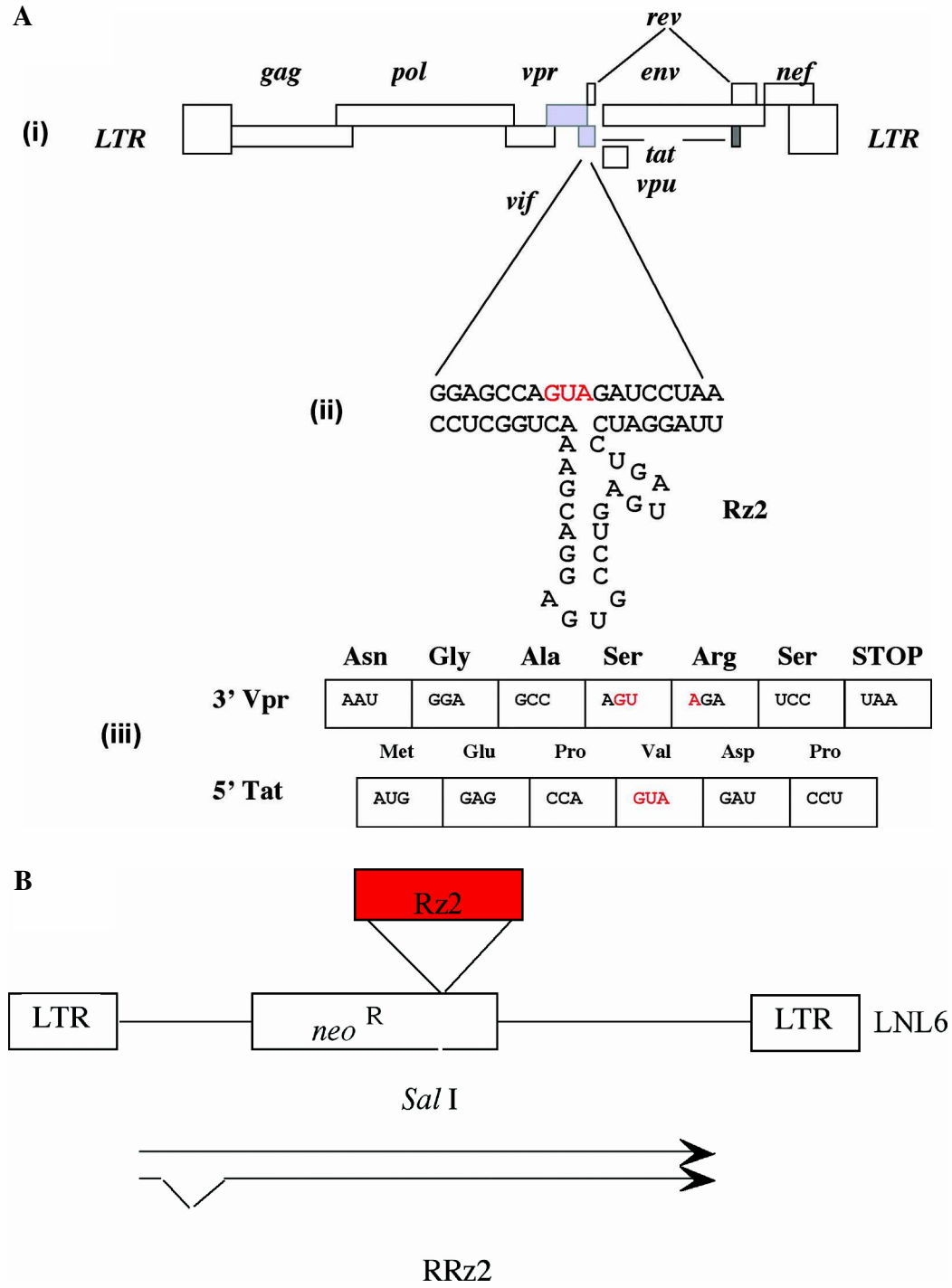

FIG. 1. Ribozyme target site and vectors used. **A**: Location of the ribozyme target site within the human immunodeficiency virus type 1 (HIV-1) genome. (**i**): Schematic of human HIV-1 genome showing location of replicative, regulatory, and accessory genes; the Rz2 target site is within *vpr* and *tat*, shown in light blue. (**ii**): Rz2 and its target sequence showing the Rz2 complementary arms and catalytic domain; the target GUA cleavage site is shown in red. (**iii**): Location of the GUA target sequence (shown in red) in *vpr* and *tat*. **B**: Vectors used in the study. Two vectors were used, LNL6 (Bender *et al*., 1987) and RRz2, which was produced from LNL6 by cloning Rz2 into the 3′ untranslated region of the neomycin phosphotransferase gene (neo^R). A spliced and nonspliced message is produced from both vectors.

for integrity of the constructs and sent to BioReliance Corporation (Rockville, MD) for manufacture of a Master Cell Bank and subsequent manufacture of virus with certified safety testing. Virus was harvested in IMDM; all batches of retroviral supernatant (LNL6 and RRz2) were tested for sterility, replication-competent retrovirus, and general safety by BioReliance Corporation. Viral titers were assessed by infecting the NIH 3T3 cell line using serial dilutions and scoring G418 resistance. LNL6 and RRz2 titers were 1.4×10^7 and 0.8×10^7 infectious viral particles per milliliter, respectively. Retroviral supernatant was added to $CD34^+$ cells once daily for 2 days for patients 1 to 7, and 3 times over 2 days for patients 8 to 10. For patients 4 to 10, transductions were performed on flasks coated with the CH296 fragment of human fibronectin (BioWhittaker, Inc., Walkersville, MD). To inhibit potential HIV replication *in vitro*, $CD34^+$ cell cultures and transductions were carried out in the presence of nevirapine at a concentration of 500 n*M* (Boehringer Ingelheim, Ridgefield, CT). Absence of HIV replication was verified by measuring p24 antigen by enzyme-linked immunosorbent assay (ELISA) in the final infusate (all 10 samples had undetectable p24 levels). Fungal, bacterial, and mycoplasma cultures, as well as endotoxin assays were negative in the final cell product for all 10 patients.

Analysis of replication competent retrovirus

Replication competent retrovirus (RCR) testing of the final cell infusate was performed in 5% of the viral supernatant at the end of transduction as well as in 1% of the transduced $CD34^+$ cells by cocultivation, using a two-passage amplification step in the *Mus dunni* cell line. The resulting *Mus dunni* cell culture supernatants were then tested for infectious retrovirus using the PG4 S+L− focus assay. For RCR detection in patient cells, DNA extracted from peripheral blood mononuclear cells (PBMC) 6 months and 1 year after transduced-$CD34^+$ cell infusion was analyzed for the presence of amphotropic envelope sequences using the following primers: CTATGTGATCTGGTCGGAGA and CCACAGGCAACTTTAGAGCA. The assay allows the detection of RCR by amplifying a highly conserved region that encodes part of the host-determining region of the envelope gene, which is required for infection of cells through the amphotropic receptor. The amplified region is 289-bp long. The sensitivity of the assay is 1 positive cell in a background of 10^5 negative cells. A positive control with the LNL6 producer cell line PA317 was run in each assay. Polymerase chain reaction (PCR) products were resolved on a 2.5% NuSieve Gel (FMC BioProducts, Rockland, ME).

Derivation and selection of hematopoietic cells for analysis of vector presence and expression

Granulocytes were selected from peripheral blood by density gradient centrifugation (Accurate Chemical & Scientific Corporation, Westbury, NY). Monocytes and T lymphocytes were selected from PBMC using the magnetic antibody cell separation (MACS) system (Miltenyi Biotech Inc., Auburn, CA) in which cells are labeled with a magnetic antibody and passed through a column resulting in a positive and negative fraction. Monocytes and T lymphocytes were separated from PBMC using CD14 and CD3 direct MicroBeads (Miltenyi Biotech Inc., Auburn, CA), respectively. T cells were also separated further into subpopulations as follows: CD4 cells were separated from PBMC using the MACS CD4 multisort bead kit. The negative fraction was then labeled with the MACS CD8 multisort bead kit. The CD4 and CD8 positive fractions were both labeled with a CD45RA antibody conjugated to fluorescein isothiocyanate (FITC) and separated using the MACS anti-FITC multisort kit. CD62L selection was then performed on the CD4/CD45RA- and CD8/CD45RA-positive fractions using a murine IgG2a anti-CD62L antibody (Becton Dickinson, Franklin Lakes, NJ), followed by selection with rat anti-mouse IgG2a+b MicroBeads. Purities were verified by flow cytometry for all subpopulations to be above 90%.

Fine-needle aspirates of inguinal or axillary lymph nodes were performed by conventional technique using a 22-gauge needle. The material was expelled from the syringe into a 15-ml vial containing 10 ml of RPMI plus 1% fetal calf serum (FCS). The cell suspension was counted; 5×10^6 cells were used for flow cytometry analysis. The remainder were used to prepare a cytospin and after flash-freezing DNA was extracted for PCR analysis.

PCR analysis of vector presence in hematopoietic cells

PCR was performed by three methods: competitive PCR, nested competitive PCR, and nested noncompetitive PCR. As a negative control in all PCR amplifications, unmarked PBMC were included, to assess for any vector contamination, in DNA extractions and subsequent PCR amplifications. Competitive PCR was used for determination of $CD34^+$ cell transduction efficiency prior to infusion; this method has been previously described (Knop *et al.*, 1999).

Nested competitive PCR was used to determine vector copy number in mature hematopoietic cells. This was performed at 4 weeks in PBMC. The range of the standard curve was from 0.01–0.00125% (or 1/10,000–1/80,000). Twelve repeats for each sample were performed in round 1. A 164-bp sequence competitor was included in each first round sample, as well as primers 5Nes1:GATCCCCTCGCGAGTTGGTTCA and 3L2A: GAGTTCTACCGGCAGTGCAAA. Annealing and denaturation temperatures were 68°C and 94°C, respectively. Subsequently, the 12 replicate samples were pooled and 5 μl of each pool included as template for round 2, where primers were 3L2A and P^{32}-labeled 5L1A: CACTCATGAGATGCCTGCAAG, at the same cycling conditions as round 1. Samples were resolved on a 5% denaturing polyacrylamide gel electrophoresis (PAGE) gel, and quantitated using Molecular Dynamics Imagequant software (Amersham Biosciences, Piscataway, NJ). Marking (%) of samples was calculated by dividing the sum of the RRz2 and LNL6 band intensities by the intensity of the competitor band. Because this method required DNA extraction from 10^7 cells, it was only used at one time point (4 weeks).

Amplification efficiencies for competitive and nested competitive PCR were assessed using 1:1 (LNL6:RRz2) samples. The third method, noncompetitive nested PCR, was used for cell marking and for assessment of expression (see next section) of the constructs. The efficiency of this nested PCR method for DNA analysis was performed on ratios 1:1, 1:3.5, 1:5, and 1:10 (LNL6:RRz2) and on the inverse ratio 10:1 (LNL6:RRz2). For analysis of specific cell phenotypes, we used

DNA from 10^6 cells. When compared to the competitive PCR assay during preclinical validation, substantially similar results were obtained. In this assay, a DNA ratio control was constructed by diluting marked CEM T4 DNA transduced with LNL6 & RRz2 at a ratio of 1:3.5 or 1:5 (where LNL6 = 1) in a background of PBL (negative) DNA to a concentration of 0.005% marked cells. Nested (hot start) PCR was performed using primers 5Nes1 and 3L2A in round 1 (10 replicates, 17 cycles) and 3L2A and P^{32}-labeled: 5L1A in round 2 (pooled replicates, 35 cycles). Conditions were as above. Quadruple product samples were resolved and quantitated as above. This assay was validated to its lower limit of detection, which was 0.001%, but for reproducibility the limit was set at 0.005%. Results were accepted if the ratio control was within accepted limits set during validation experiments (i.e., for a ratio of 1:3.5 LNL6:RRz2 in a 0.005% vector-containing sample, accepted range was 1:1.1 to 1: 6.9). Only PCR results in which the control fell within this range were accepted. We estimated copy number by comparing radioactivity volumes to these controls consisting of 1/25,000, 1/100,000 (for 1:5 dilution controls), and negative control. Before each band was used as an independent control we verified that both vectors amplify with equal efficiency.

Assessment of vector expression in hematopoietic cells

The third method, noncompetitive nested PCR, was also used for analysis of construct expression. RNA was prepared from PBMC selected by Ficoll-Hypaque centrifugation of blood samples, using the Qiagen RNeasy kit (Valencia, CA), following the manufacturer's instructions. Residual DNA was removed by DNase digestion. RNA was reverse-transcribed using Gibco Superscript Reverse Transcriptase (Carlsbad, CA) with 7 replicates of each sample. Samples were then pooled, and cDNA amplification was performed on 10 replicates as described above. Expression levels in patient samples were consistently detected at levels within the range of the control dilutions.

Statistical analysis

Spearman rank correlation was used to quantify the relationship between the number of transduced $CD34^+$ cells infused and subsequent gene detection at 2 years postinfusion of progeny PBMC and T lymphocytes. Analyses are based on values given in transduced $CD34^+$ cells per kilogram for each patient. In these analyses, LNL6 marking is correlated with the quantity of LNL6-transduced cells reintroduced, and RRz2 marking is correlated with the quantity of RRz2-transduced cells reintroduced.

Analysis of ribozyme cleavage sites

Ribozyme cleavage site analysis was done as previously described (Wang *et al.*, 1998) with minor modifications. Briefly, viral RNA was extracted and reverse transcribed with the Access RT-PCR kit (Promega, Madison, WI) using primer 1: TGGCAATGAAAGCAACACT for 45 min at 48°C. The resulting cDNA was PCR-amplified by addition of primer 2: TTTAGAGGAGCTTAAGAATGA for 25 cycles (94°C for 20 sec, 55°C for 30 sec, and 68°C for 30 sec). Single-stranded DNA for cycle sequencing was produced by a second PCR step using AmpliTaq (Perkin-Elmer, Boston, MA) and primer 3: AGTTTTAGGCTGACTTCCTGG for 25 cycles (94°C for 20 sec, 55°C for 30 sec, and 68°C for 30 sec). Sequencing was performed on purified PCR products with the ABI PRISM Dye termination cycle sequencing Ready Reaction kit with AmpliTaq DNA polymerase (Perkin-Elmer) on an automated DNA sequencer (ABI Model 377, Applied Biosystems, Foster City, CA) using primer 4: TGGAAGCCATAATAAGAAT. Sequence alignment was performed with Sequence Navigator software (Perkin-Elmer) and manually proofread and edited. The resulting sequence was compared to the HXB2 clade B HIV-1 reference strain.

RESULTS

Adverse events and feasibility

This phase I gene transfer study sought to analyze the gene transfer procedure and its feasibility. The administration of G-CSF, apheresis, and reinfusion procedure was reasonably well tolerated with the following possibly or probably related

TABLE 1. SUMMARY OF PATIENT CHARACTERISTICS AND OF THE $CD34^+$ HPC INFUSION PRODUCT

Patient	*Age*	*Gender*	*ART*	*Retronectin*	*$CD34^+$ Purity (%)*	*Infused $CD34^+$ cells ($\times 10^6$/kg)*	*Transduction (%)*	*Transduced $CD34^+$ cells ($\times 10^6$/kg)*
01	59	M	1	No	65	3.38	0.4	0.01
02	44	M	4	No	80	2.08	4	0.08
03	40	M	4	No	66	2.98	2	0.06
04	44	M	6	Yes	67	1.29	10	0.13
05	37	M	6	Yes	94	10.01	7	0.70
06	32	M	3	Yes	90	1.63	32	0.52
07	41	M	3	Yes	96	8.45	48	4.06
08	46	M	2	Yes	98	9.37	57	5.34
09	48	M	3	Yes	93	1.64	36	0.59
10	38	F	1	Yes	95	5.07	28	1.42

The table shows patient, age, gender, number of prior antiretroviral regimens (ART), use of retronectin (RN) to support transduction, $CD34^+$ purity, number of infused $CD34^+$ cells, percentage transduction, and number of transduced $CD34^+$ cells infused.
HPC, hematopoietic progenitor cells.

adverse events. These were: pre-cell infusion: bone and muscle pain (5 patients classified as: mild, 3; moderate, 1; and severe, 1); and on or after cell infusion: nausea, sweating, and hypotension (1 patient each classified as mild); thrombocytopenia (3 patients classified as mild); transient symptoms resembling upper respiratory tract infection (5 patients classified as mild); and diarrhea (4 patients classified as mild). There were 2 serious adverse events possibly or probably related and occurring before or during cell infusion: 1 patient with musculoskeletal connective tissue and bone pain and 1 patient with severe anxiety; while a third serious adverse event (nonspecific gastroenteritis post-cell infusion) was not considered to be caused by the study drug or study procedure. These results indicate that the procedure is reasonably well tolerated.

In terms of feasibility we sought to assess whether (1) the in-

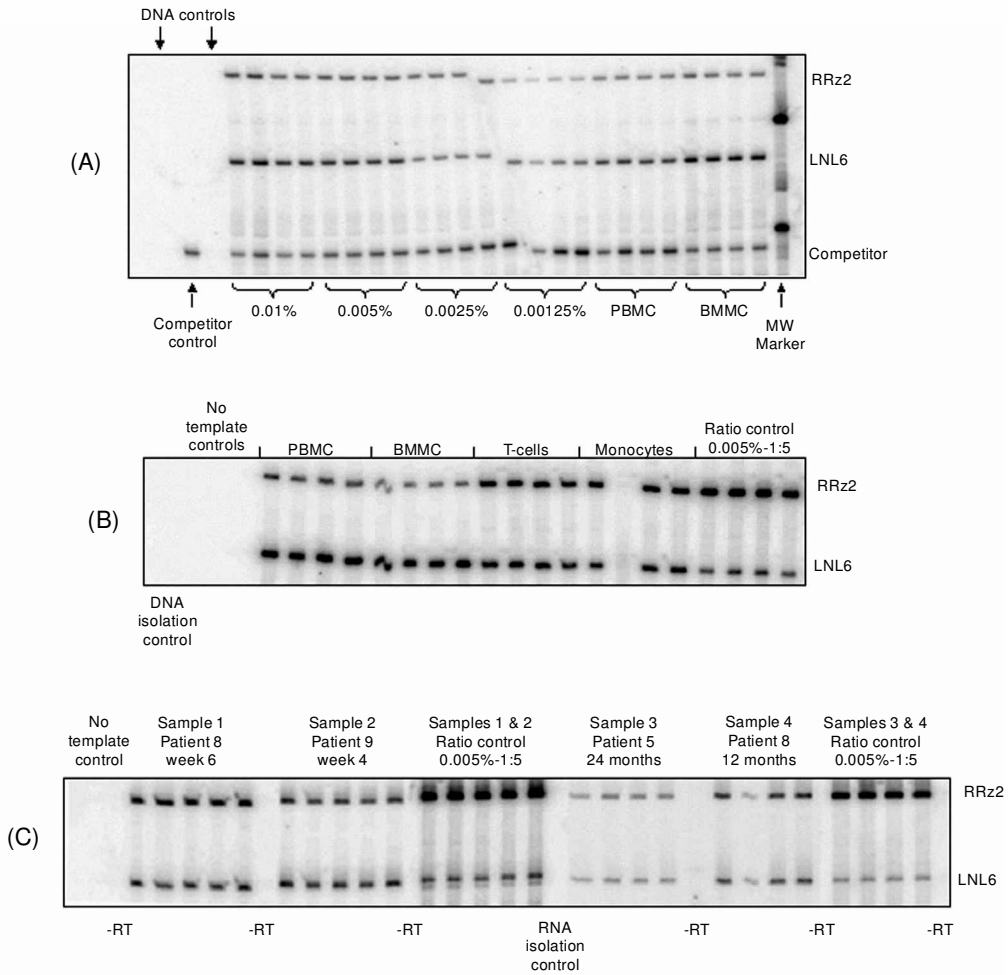

FIG. 2. Long-term vector presence and expression in hematopoietic cell subsets. Polymerase chain reaction (PCR) analysis was performed in leukocyte subsets to determine vector copy frequency using primers directed against the *neo*^{R} gene that span the Rz2 sequence in the RRz2 vector. PCR products for LNL6 and RRz2 are 174 and 216 bp, respectively, and include a stretch of the untranslated terminus of the *neo*^{R} gene. As a negative control in all PCR amplifications, unmarked peripheral blood mononuclear cells (PBMC) were included, to assess for any vector contamination, in DNA extractions and subsequent PCR amplifications. No template controls were PCR bulk mix alone (no cellular DNA). **A**: Nested competitive PCR. LNL6 and RRz2 vector sequences as well as a band corresponding to a competitor sequence used for DNA quantification are shown in the first gel in PBMC, and bone marrow mononuclear cells (BMMC) from patient 7. Amplification efficiencies for nested competitive and competitive PCR were conducted at 1:1 (LNL6:RRz2) samples (see Study Design). **B**: Noncompetitive nested PCR. Detection in PBMC, BMMC, T-lymphocytes, and monocytes in patient 5 two years after infusion of transduced CD34$^+$ cells is shown using the noncompetitive PCR method. T lymphocytes and monocytes were selected from PBMC to a purity $> 90\%$, as confirmed by flow cytometry. For each cell type, four replicates of a pool of samples are shown as described in Materials and Methods. **C**: Noncompetitve nested reverse transcriptase-polymerase chain reaction (RT-PCR). Short- and long-term expression of both LNL6 and RRz2 in PBMC is shown for three representative patients (patients 5, 8, and 9), as measured by RT-PCR. Expression was assessed in a reverse-transcriptase (RT+) nested PCR using radiolabeled primer. For each sample, a reaction that did not contain reverse-transcriptase (RT−) was included. Quantification was achieved by comparing radioactivity levels between samples and controls. The examples shown here have similar intensities to the LNL6 control bands of the 1:5 ratio controls, thus the approximate amount of vector RNA detected is 0.005%.

troduction of an anti-HIV–1 ribozyme into hematopoietic progenitor cells could result in the emergence of thymic emigrants bearing vector sequences, (2) normal T-lymphocyte and myeloid maturation could take place in genetically modified cells, (3) vector presence and expression could persist long-term and (4) whether there was any evidence for preferential survival of ribozyme-containing cells. The data in the sections that follow show that (with the exception of definitive evidence for preferential survival) each of these parameters related to feasibility of approach were met.

Transduction of CD34$^+$ cells and patient infusion

Ten patients were enrolled in this study (Table 1). The mean age was 43 years (range, 32–59). The median number of antiretroviral regimens used was 3 (range, 1–6). The total number of CD34$^+$ cells infused ranged from 1.3–10.1 $\times$ 10^6 cells per kilogram of body weight (median, 3.2 $\pm$ 1.1 $\times$ 10^6 cells per kilogram). Transduction efficiency in the first 3 patients was low (range, $<$ 1%–4%), accounting for the number of transduced CD34$^+$ cells infused ranging from 0.01–0.08 $\times$ 10^6 cells/kg (Table 1). Cells carrying the transgene were detected up to 6 months in patient 1 (ribozyme in bone marrow and PBMC, LNL6 in granulocytes), up to 9 months in patient 2 (RRz2 in PBMC), and 12 months in patient 3 (LNL6 in PBMC and RRz2 in monocytes). To improve transduction efficiency, 7 subsequent patients received autologous CD34$^+$ cells transduced in the presence of the CH296 fragment of human fibronectin (Hanenberg *et al.*, 1996). In these patients, transduction efficiency increased to a median level of 32 $\pm$ 6.9% (range, 7%–57%). On average, transduction efficiency for LNL6 was 1.6 times higher than that obtained with RRz2, probably reflecting the difference in vector titer (see Materials and Methods). Transduction efficiencies were also determined by performing PCR for vector sequences in single colonies grown from the final transduced CD34$^+$ cell product. Both methods showed consistent results. In all 10 patients, biologic assays RCR in CD34$^+$ cells at the end of transduction using both viral supernatant and cultured CD34-enriched cells were negative. Patient PBMC samples analyzed by PCR for RCR 6 months and 1 year after CD34$^+$ cell infusion also revealed no evidence of RCR.

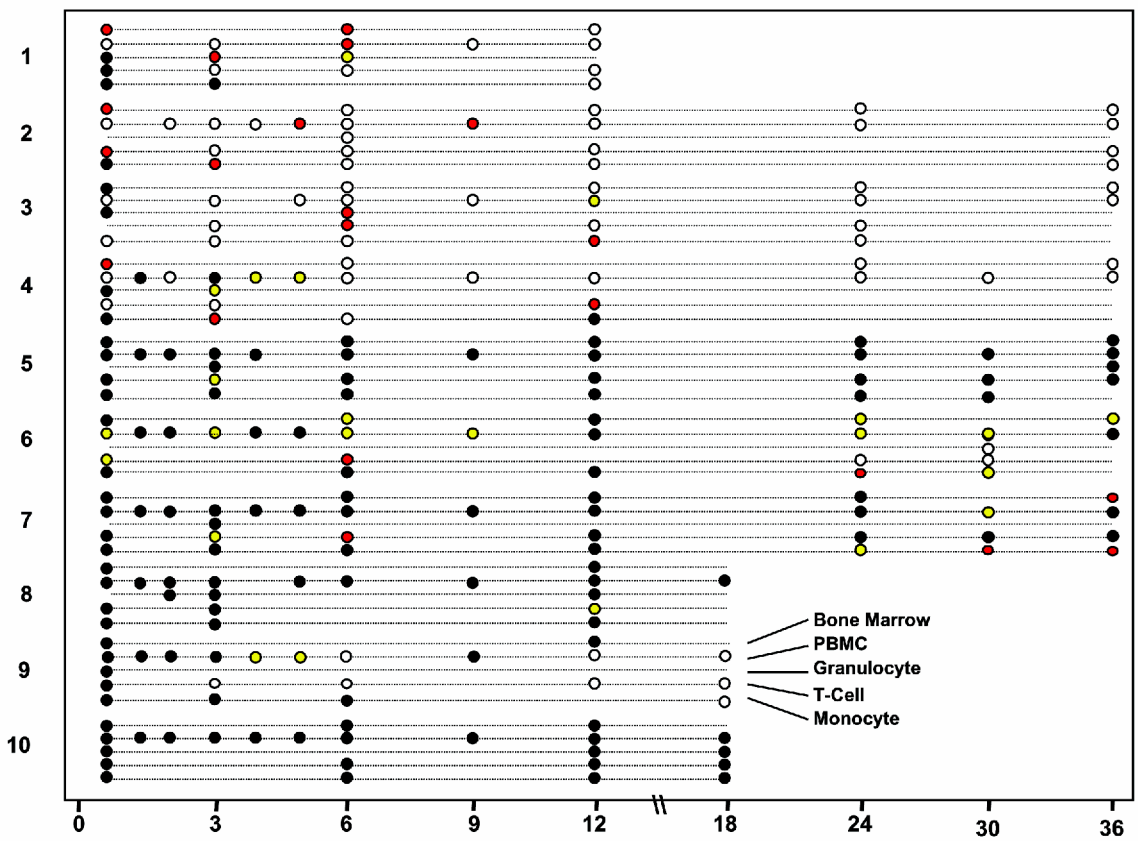

FIG. 3. Summary of vector detection by polymerase chain reaction (PCR) in all patients. Cells were examined by PCR for LNL6 or RRz2 vector detection up to 36 months postinfusion. Cell types were bone marrow mononuclear cells (BMMC), peripheral blood mononuclear cells (PBMC), granulocytes, T lymphocytes, and monocytes as indicated. Data are shown for each patient as labeled in the Y-axis with time on the X-axis. Longer term gene marking was observed after the use of the fibronectin fragment (CH296), which resulted in an increase in transduction efficiency (see Table 1). The presence or absence of vector detection is indicated by colored circles, without regard to vector copy number; the percentage of gene containing cells was in the range $1/10^4$ to $1/10^5$: yellow: LNL6 detected, red: RRz2 detected, black: both vectors detected, open: neither vector detected. The design of presentation was first used by Emmons and Dunbar (1997).

Analysis of multilineage vector persistence and expression and correlation with CD34$^+$ cell dose

After a median follow-up of 30 months (range, 12–36 months), transgenes were detected in all patients at multiple time points and in multiple hematopoietic lineages. On average, gene presence was found in $1/10^4$ to $1/10^5$ of hematopoietic cells analyzed. Figure 2A shows quantification of vector copy number in patient 7 showing vector presence in 0.01% of PBMC and bone marrow mononuclear cells (BMMC) 4 weeks postinfusion. Figure 2B shows long-term multilineage gene presence in a representative patient 5. Figure 3 shows a summary of detection of both ribozyme and control vector transgene by nested PCR in BMMC, PBMC, granulocytes, T lymphocytes, and monocytes in all 10 patients up to 3 years postinfusion. For both vectors, we found a strong linear correlation between the number of transduced CD34$^+$ cells infused and the persistence of gene detection at 2 years postinfusion in both PBMC (LNL6 $p = 0.021$; RRz2 $p = 0.034$) and T-lymphocytes ($p < 0.0001$ for both LNL6 and RRz2). The minimum number of transduced CD34$^+$ cells that resulted in marking longer than 1 year was 0.5×10^6 cells per kilogram. We also analyzed expression of the gene constructs in PBMC using semiquantitative reverse transcriptase-polymerase chain reactin (RT-PCR). Figure 2C shows vector expression up to 2 years postinfusion in 3 representative patients.

We analyzed fine-needle aspirates of lymph nodes from 4 patients for the presence of vector sequences. Both LNL6 and RRz2 were detected in 2 of the 4 patients (patient 7 at 2.5 years postinfusion and patient 10, 1 year postinfusion; data not shown).

Analysis of vector persistence in naïve T lymphocytes

To determine whether transduced CD34$^+$ cells could undergo T-lymphocyte development in HIV-infected patients we selected peripheral blood CD4$^+$ and CD8$^+$ cells for CD45RA and CD62L surface marker expression, which characterize naïve T lymphocytes (Sanders *et al.*, 1988; Picker *et al.*, 1993), and we analyzed these subpopulations for the presence of LNL6 and RRz2. Vector sequences were detected in naïve cells (> 90% CD45RA$^+$/CD62L$^+$) up to 3 years postinfusion (the last time point evaluated). Figure 4 shows vector sequences in highly enriched naïve and memory cells from 4–130 weeks postinfusion in 3 patients. Because of the numbers of gene-marked cells present and the limit of detection of the nested noncompetitive PCR (see Materials and Methods) in this case (and others), the bands must be amplified from the greater than 90% pure naïve or memory cells; the cell numbers in the remaining cell population(s) are not sufficient to yield detectable signals (see Study Design). The reason for this is that the analysis is at the limit of detection with the 10 μg of DNA used in the assay. Thus, the signal from any contaminating cell population will be below the level of detection, and for the signal to come from the contaminating cells only (< 10% cell population and often this was < 5%) it would have to be present in a percentage of the contaminating cells that exceeds that obtained in the various cell populations in the total study. The average age and viral load of patients whose naïve T lymphocytes had detectable vector sequences were 43 years (range, 32–59 years) and 3680 copies per milliliter (range, undetectable to 22,628 copies per milliliter), respectively. There did not appear to be any age-related or ART dependence in any of these parameters. A summary of vector (RRz2 and LNL6) detection in naïve T lymphocytes at the time of detection is shown in Table 2.

Analysis of viremia and circulating CD4$^+$ T-lymphocyte counts

All patients in this phase I study have been receiving ART (generally a combination of nucleoside reverse transcriptase inhibitors and protease inhibitors) and to date, none of the patients have developed opportunistic infections. The average change in CD4$^+$ T-lymphocyte count from entry to year 3 was an increase of 10 cells/mm^3 (range, -40 to $+80$). An initial increase in viral load was observed at day 1 postinfusion in some patients who discontinued ART during the period of mobilization. Drug discontinuation or substitution of nonnucleoside reverse transcriptase inhibitors for nucleoside reverse transcriptase inhibitor or protease inhibitor was included in the protocol to prevent potential inhibition of MoMLV reverse transcriptase during transduction (Bazin *et al.*, 1989). Viral load decreased by an average of 2.25 logs in 6 patients (0.35–3.9), remained undetectable in 3 patients, and increased by 1 log in one patient. Rises in viremia responded to modifications of antiretroviral therapy. The mean viral loads (copies per milliliter) for each patient were: patient 1, 644; patient 2, 6,808; patient 3, 899; patient 4, 15,524; patient 5, 10,929; patient 6, 2,643; patient 7, 966; patient 8, 96; patient 9, 35; patient 10, 893. Changes in viremia or CD4$^+$ T-lymphocyte counts did not correlate with the persistence of vector detection or vector expression in any cell type, and are thought to be influenced by individual viral susceptibility to ART.

Genotypic analysis for viral resistance to Rz2

Viral genotyping demonstrated multiple drug resistance mutations in all patients (data not shown). We also performed genotypic analysis of the Rz2 binding/cleavage region of HIV in all patients at study entry and at 12, 24, 52, and 104 weeks posttreatment. Six of the 10 patients had sufficient viral loads to permit sequence determination. Patients 1, 2, 5, and 6 had wild-type sequences. Patient 4 had an A to C transition at position -1 from the GUA target triplet. Patient 7 had a G to T transition at position -4 from the GUA target triplet. Neither of these single mutations was within the GUA triplet, and *in vitro* cleavage reaction showed that the Rz2 ribozyme was active against these target sequences (data not shown). The mutations detected in both these patients were present before treatment also indicating that they did not arise as a result of efficacy induced resistance to the construct.

DISCUSSION

The current study has shown that gene constructs can be retrovirally introduced into CD34$^+$ hematopoietic progenitor cells, and that these cells will contribute long-term to multilineage hematopoiesis in adult HIV-infected patients. These studies were conducted in the absence of myelosuppression, therefore the engineered CD34$^+$ cells contributed to form a chimeric hematopoietic system. Because no survival advantage is ex-

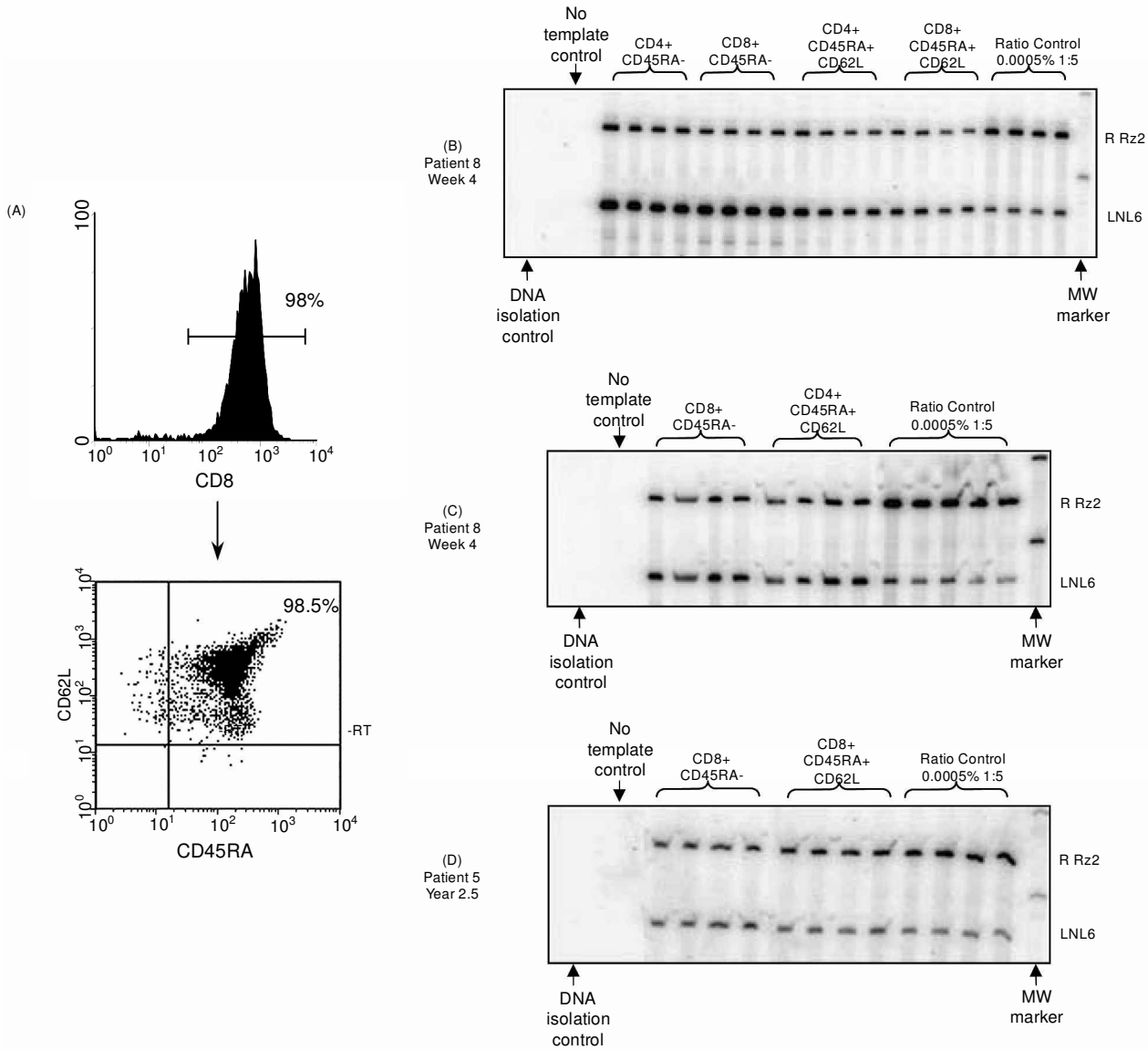

FIG. 4. Example of naïve T cell subset purity and detection of vector sequences in T cells. Representative flow cytometry histogram and plot after selection of peripheral blood mononuclear cells (PBMC) for CD8, CD45RA, and CD62L expression in patient 5 (**A**). The gels presented show polymerase chain reaction (PCR) results for LNL6 and RRz2 vector sequences in $CD4^+$ and $CD8^+$ T lymphocytes and in naïve T-lymphocytes subsets selected from peripheral blood in three patients. Vector sequences were detected in naïve T-cell subsets as early as 4 weeks postinfusion (**B**). Long-term detection is also shown at 2 years (**C**) and 2.5 years (**D**) after infusion of transduced $CD34^+$ cells. As a negative control, unmarked PBMC were included to assess for any vector contamination in DNA extractions and subsequent PCR amplifications. No template controls were PCR bulk mix alone (no cellular DNA).

pected to occur at the level of the transduced $CD34^+$ cells, and given our results showing a correlation between persistence of vector-containingperipheralblood cells and numbers of infused $CD34^+$ cells, we believe that future studies should aim to increase the number of gene modified cells administered. In the first report of gene therapy efficacy in a human disease, it was reported that genetic correction of the γc cytokine receptor deficiency that characterizes human severe combined immunodeficiency (SCID)-X1 disease leads to the development of a functional immune system (Cavazzana-Calvo *et al.*, 2000) albeit with the potential in that case for the subsequent development of leukemia as a result of insertional mutagenesis (Hacein-Bey-Abina *et al.*, 2003). There are several key differences in the SCID-X1 study that may impact on the frequency of insertional mutagenesis leading to cancer. These include age of patient and phenotypic immaturity of the progenitor cell popoulation, the proliferation-inducing γ common chain transgene and the disease background. It is relevant that the Report from the American Society of Gene Therapy: Ad Hoc Committee on Retroviral-Mediated Gene Transfer to Hematopoietic Stem Cells

Table 2. Detection of Vector in Naïve T Lymphocytes

Patient number	*<3 months*	*3–6 months*	*6–12 months*	*12–24 months*	*>24 months*
1	NT	NT	NT	NT	NT
2	NT	NT	R	NT	NT
3	NT	NT	NT	NT	NT
4	L	NT	—	NT	NT
5	NT	NT	L	L/R	L/R
6	NT	R	NT	NT	NT
7	L/R	NT	L/R	L/R	L/R
8	L/R	NT	NT	NT	NT
9	L	NT	R	R	NT
10	NT	L/R	L/R	NT	NT

Circulating naïve T-lymphocyte populations (either $CD4^+CD45RA^+CD62L^+$ or $CD8^+CD45RA^+CD62L$) were isolated from $CD4^+$ or $CD8^+$ selected cell populations to >90% purity from several patients at various times postinfusion, and analyzed by PCR as described in Figure 4. The presence of the vector sequences is indicated as follows: R = RRz2 only, L = LNL6 only, L/R = both RRz2 and LNL6 detected, 0 = neither RRz2 nor LNL6 detected, — = not tested or insufficient DNA (per parameters in Materials and Methods) for meaningful data. In each case, the percentage of gene containing cells was in the range $1/10^4$ to $1/10^5$.

PCR, polymerase chain reaction.

(April 2003), has identified no other cases of retroviral insertional mutagenesis leading to cancer in human gene transfer clinical trials to date.

With regard to the application of gene correction strategies, the HIV-infection model differs substantially from the SCID-X1 model. In SCID-X1 gene correction, where the resulting functional receptor mediates survival signals, thymopoiesis results only from $CD34^+$ cells that contain the exogenous gene. In contrast, in HIV/AIDS both transduced and nontransduced $CD34^+$ cells can contribute to thymopoiesis and a survival advantage (protection from HIV) would occur at the level of the $CD4^+$ T-lymphocyte potentially resulting in an expansion of these ribozyme-containing cells. In this case, the HIV provides the selective survival pressure, as unprotected cells would remain vulnerable. This hypothesis was not tested adequately in our study because the patients have remained on ART, the numbers of gene marked cells in the peripheral blood is relatively low, and high levels of viral replication were treated quickly with modifications in their drug regimens. In terms of preferential survival, a statistically significant slower decay of RRz2 relative to LNL6-containing T lymphocytes was observed (-0.0001 copies per month for RRz2 versus -0.0175 copies per month for LNL6; difference $p < 0.001$). No differences in decay rates were observed in granulocytes and other cell populations not susceptible to HIV infection (e.g., granulocytes: -0.024 copies per month for RRz2 versus -0.034 copies per month for LNL6; difference $p = 0.728$). A separate analysis that sought to adjust for initial differences in the number of RRz2-transduced $CD34^+$ cells infused by expressing the results as a percentage of initial lymphocyte marking (more LNL6-transduced $CD34^+$ cells were infused presumably as a result of the higher titer of LNL6 versus RRz2), showed a trend in terms of preferential survival that was not significant ($p = 0.132$) in terms of numbers of vector-containing T-lymphocytes. Thus, while we have shown feasibility in terms of progenitor cell transduction and engraftment, definitive conclusions regarding preferential survival and efficacy would likely require a greater number of transduced progenitor cells. As seen in adenosine deaminase deficiency (Aiuti *et al.*, 2002), it is also possible that a higher degree of transduced progenitor cell engraftment and a degree of preferential survival leading to clinical benefit could occur after the induction of bone marrow suppression. The lack of survival advantage at the level of hematopoietic progenitors that is inherent to HIV infection likely explains the low level of gene presence in mature cells observed in the present study.

We cannot state with certainty whether our results are derived from true pluripotent hematopoietic stem cell transduction versus transduction of a precursor population. Previous published reports have shown that integration studies in progenitor cell gene therapy generally show a polyclonal pattern (Kohn *et al.*, 1998; Cavazzana-Calvo *et al.*, 2000), probably reflecting the contribution to hematopoiesis of multiple precommitted precursors. The long-term nature of vector presence argues that early precursors or true progenitor cells were transduced in this study. We have detected colony-forming units bearing vector sequences that are resistant to G418 (indicating neomycin phosphotransferase protein expression) up to 2 years postinfusion, probably reflecting sustained production of myeloid precursors from transduced stem cells. We have discounted that our results are the consequence of transduction of mature cells present in the infusion cell product, as we continue to see gene presence in short-lived cells such as granulocytes and monocytes for over 140 weeks after infusion.

This study represents the second report of an HPC gene therapy trial in HIV infection. A previous trial using a retroviral vector containing a *rev*-responsive element decoy gene in pediatric patients resulted in detection of the anti-HIV gene and control vector in PBMC in 2 of the 4 patients only at day 1 after cell infusion. Control vector was detected at low levels up to 330 days postinfusion in one patient (Kohn *et al.*, 1999). Based on our results, the short-term marking observed in the previous report seems to be due at least in part to low doses of trans-

duced $CD34^+$ cells administered. Whereas previous HIV gene therapy studies using transduced mature T lymphocytes have shown longer persistence of a therapeutic vector compared to a control vector up to a year after infusion (Ranga *et al.*, 1998), this is the first report to indicate that T-lymphocyte development can ensue long-term from genetically modified hematopoietic progenitors in the context of HIV infection. The presence of vector-containing naïve T cells indicates that successful engraftment of the $CD34^+$ progenitor cells and consequent thymopoiesis occurs in adult HIV-infected patients. The presence of vector-containing granulocytes and monocytes, indicates successful myelopoiesis after engraftment. The finding that sustained production of transgene-containing naïve T lymphocytes occurs even in patients with detectable viremia is significant, given that HIV can infect naïve thymocytes (Ostrowski *et al.*, 1999), and that the thymus can act as a source of HIV-1 latency during T-lymphocyte differentiation (Brooks *et al.*, 2001). As thymopoiesis continues in the adult patient (Jamieson *et al.*, 1999; Poulin *et al.*, 1999; Smith *et al.*, 2000), replacement of this naïve T-lymphocyte–based latent pool with cells that are engineered to inhibit HIV replication effectively could result in restoration of protected immune cells. It appears that unlike other studies using hygromycin/thymidine kinase transgene (Riddell *et al.*, 1996) the neo^R transgene does not elicit an immune response leading to the elimination of the gene modified cells. The presence of ribozyme sequences in monocytes/macrophages could also contribute to control viral reactivation in this latent pool (Koenig *et al.*, 1986; McElrath *et al.*, 1989; Zhu *et al.*, 2002), which is known to play a significant role in late-state infection (Orenstein *et al.*, 1997; Igarashi *et al.*, 2001). Although proof of clinical efficacy would require further human experimentation, the results presented herein justify further exploration of anti-HIV hematopoietic progenitor cell gene transfer as a form of anti-HIV therapy.

ACKNOWLEDGMENTS

We thank the patients who took part in this study. We thank Alison Knop for her help with trial organization; John Murray for his assistance with statistical analysis; Fiona Elliot and Allison Arndt for technical assistance; and Irvin Chen and Wayne Gerlach for helpful manuscript review. We thank Amgen Inc. and Boehringer Ingelheim Corporation for providing valuable reagents. We acknowledge support by the Gene Therapy, Virology, and Flow Cytometry Cores of the UCLA AIDS Institute CFAR grant #AI28697, and by the UCLA General Clinical Research Center Grant MO1-RR-00865. R.G.A. is supported by NIH/NIAID award #AI01662. R.T.M. is supported by USPHS NIH grants AI27660, CA70080, and by a grant from the State of California University-wide AIDS Research Program (CC99-LA-002). This clinical trial was supported by funds from Gene Shears Pty. Limited, and Johnson & Johnson Research Pty. Limited.

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Received for publication June 1, 2003; accepted after revision December 3, 2003.

Published online: February 18, 2004.