HUMAN GENE THERAPY 10:2505–2514 (October 10, 1999)
Mary Ann Liebert, Inc.

Inhibition of Human Immunodeficiency Virus Replication and Growth Advantage of CD4$^+$ T Cells and Monocytes Derived from CD34$^+$ Cells Transduced with an Intracellular Antibody Directed against Human Immunodeficiency Virus Type 1 Tat

MARK C. POZNANSKY,[1] JOYCE LA VECCHIO,[2] SANDRA SILVA-ARIETTA,[1] JULIE PORTER-BROOKS,[3] KATE BRODY,[1] IVONA T. OLSZAK,[1] GREGOR B. ADAMS,[1] URBAN RAMSTEDT,[3] WAYNE A. MARASCO,[2] and DAVID T. SCADDEN[1]

ABSTRACT

Current clinical gene therapy protocols for the treatment of human immunodeficiency virus type 1 (HIV-1) infection involve the *ex vivo* transduction and expansion of CD4$^+$ T cells derived from HIV-positive patients at a late stage in their disease (CD4$^+$ cell count $<$400 cells/mm^3). We examined the efficiency of transduction and transgene expression in adult bone marrow (BM)- and umbilical cord blood (UCB)-derived CD34$^+$ cells induced to differentiate into T cells and monocytes *in vitro* with an MuLV-based vector encoding the neomycin resistance gene and an intracellular antibody directed against the Tat protein of HIV-1 (sFvtat1-C$_\kappa$). The expression of the marker gene and the effects of antiviral construct on subsequent challenge with monocytotropic and T cell-tropic HIV-1 isolates were monitored *in vitro* in purified T cells and monocytes generated in culture from the transduced CD34$^+$ cells. Transduction efficiencies of CD34$^+$ cells ranged between 22 and 27%. Differentiation of CD34$^+$ cells into T cells or monocytes was not significantly altered by the transduction process. HIV-1 replication in monocytes and CD4$^+$ T cells derived from CD34$^+$ cells transduced with the intracellular antibody gene was significantly reduced in comparison with the degree of HIV replication seen in monocytes and CD4$^+$ T cells derived from CD34$^+$ cells transduced with the neomycin resistance gene alone. Further, T cells and monocytes derived from CD34$^+$ cells transduced with the intracellular antibody gene were demonstrated to express the sFvtat1-C$_\kappa$ transgene by RT-PCR and had a selective growth advantage in cultures that had been challenged with HIV-1. These data demonstrate that sFvtat1-C$_\kappa$ inhibits HIV-1 replication in T cells and monocytes developing from CD34$^+$ cells and supports the continuing development of a stem cell gene therapy for the treatment of HIV-1 infection.

OVERVIEW SUMMARY

This study examined the transduction of bone marrow- and umbilical cord blood-derived CD34$^+$ hematopoietic progenitor cells with an MuLV vector encoding an anti-HIV intracellular antibody (sFvtat1-C$_\kappa$). Transduced CD34$^+$ cells were cocultured with thymic stroma in order to facilitate T cell and monocyte differentiation. CD4$^+$ T cells and monocytes generated from CD34$^+$ cells transduced with the transgene encoding the anti-Tat intracellular antibody demonstrated expression of both a marker gene and the anti-HIV transgene. T cells and monocytes derived from CD34$^+$ hematopoietic cells transduced with the anti-Tat intracellular antibody were shown to have significantly lower levels of HIV replication and increased viability after challenge with T cell or monocytotropic isolates of HIV-1 in comparison with T cells and monocytes generated from untransduced CD34$^+$ cells or CD34$^+$ cells transduced with the LN vector alone. These data provide proof of principle for a CD34$^+$ hematopoietic stem cell-based gene therapy for

[1]Partners AIDS Research Center and MGH Cancer Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129.
[2]Division of Cancer Immunology and AIDS Research, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA 02115.
[3]Virus Research Institute, Inc., Cambridge, MA 02129.

HIV infection in which a murine retroviral vector encoding an intracellular antibody directed against HIV-1 Tat protein is used.

INTRODUCTION

HUMAN HEMATOPOIETIC PROGENITOR CELLS (HPCs) have been proposed as a target for gene therapy approaches to the treatment of a variety of diseases associated with congenital or acquired genetic defects such as human immunodeficiency virus (HIV) infection and leukemia (Belmont *et al*., 1988; Karlsson, 1991; Moritz *et al*., 1993; Nabel *et al*., 1994; Yu *et al*., 1994; Brenner *et al*., 1995; Dunbar, 1996). Gene therapy targeting HPCs offers the opportunity of repopulating the hematopoietic system with cells bearing "corrected" genetic material or encoding a transgene that protects the cell from infection or transformation (Karlsson, 1991; Brenner *et al*., 1995; Dunbar, 1996). The obstacles to the achievement of a clinically relevant hematopoietic stem cell gene therapy are many and include the introduction of a transgene that does not alter the growth or differentiation potential of the HPC and that achieves stable integration of the transgene into the target cell genome and consequently stable expression of the transgene within the differentiated cell population (Lu *et al*., 1994; Poznansky *et al*., 1998a).

HPC-based gene therapy has been proposed as a potential approach to the treatment of HIV infection (Yu *et al*., 1994). $CD34^+$ hematopoietic progenitor cells from HIV-positive patients would be transduced with a vector encoding an anti-HIV gene and then stimulated to differentiate into T cells that would be protected from HIV infection as a result of the expression of the anti-HIV transgene. This approach to the treatment of HIV infection poses its own specific problems. These include the choice of the appropriate anti-HIV gene, the effective transduction of progenitor cells that will ultimately generate T cells and other known immune cells that are infectable by HIV-1 and the expression of the relevant transgene in hematopoietic cells. Studies to date have shown in both human and rhesus models, *in vitro*, that constructs such as a polymeric *trans*-activation response (TAR) decoy, the dominant negative mutant of the Rev protein of HIV (Rev m10), or a ribozyme directed against the CAP-binding site of HIV RNA when transduced into $CD34^+$ cells could subsequently generate $CD4^+$ T cells and monocytes that were resistant to infection with HIV-1 (Yu *et al*., 1995; Rosenzweig *et al*., 1997).

We have demonstrated that primary T cells from HIV-positive patients could be transduced with a gene encoding an intracellular antibody directed against Tat or gp120 from HIV-1 and that these cells could be efficiently protected from challenge with HIV infection (Poznansky *et al*., 1998b). A phase 1 clinical trial of the *ex vivo* transduction of T cells from HIV-infected individuals with the transgene encoding the intracellular antibody against HIV-1 Tat is currently in development to determine whether this approach would be safe and effective *in vivo* (Marasco *et al*., 1998; Mhashilkar *et al*., 1999). Furthermore, it has been demonstrated that it is possible to generate $CD4^+$ T cells and monocytes that are infectable with HIV-1 in a coculture system of human $CD34^+$ HPCs with fetal human thymic stroma (Freedman *et al*., 1995; Rosenzweig *et al*., 1996). These T cells appear to have a normal T cell receptor (TCR) repertoire, proliferate in response to mitogens, and possess other features of mature lymphocytes. In this study we utilize this system to examine whether the transduction of $CD34^+$ HPCs with the transgene encoding a highly active intracellular antibody directed against the HIV Tat protein in coculture with human fetal thymic stroma results in the generation of $CD4^+$ T cells and monocytes that are protected from HIV infection.

MATERIALS AND METHODS

Retroviral vectors and packaging cell culture

LN-based murine leukemia virus (MuLV) retroviral vectors were used in all experiments (see Fig. 1) (Miller, 1992). The vector LN.sFvtat1-C_κ encoded the intracellular antibody Ln.sFv tat1-C_κ, which binds HIV-1 Tat in the cytoplasm (Marasco *et al*., 1993; Mhashilkar *et al*., 1995, 1999). The production of RNA encoding the intracellular antibody is driven by the cytomegalovirus (CMV) promoter in this vector. The LN and LN.sFvtat1-C_κ vectors also encode the neomycin resistance gene from the 5′ long terminal repeat (LTR) of the MuLV-based vector. The PG13 MuLV retroviral vector packaging cell line expressing the gibbon ape leukemia virus envelope was used and cultured by standard methods and selected in the presence of G418 (0.8 mg/ml) (Kotani *et al*., 1994; Yamada *et al*., 1994). The titer of vectors generated from the PG13 packaging cells varied between 5×10^6 and 1×10^7 infectious units/ml. Packaged vectors were filtered through 0.45-μm pore size filters and titered by limiting dilution onto Jurkat cells, which were subsequently grown in the presence of G418 at a concentration of 0.8 mg/ml. Titered vector stocks were applied to $CD34^+$ HPCs at a multiplicity of infection (MOI) of 100 as previously described (Rosenzweig *et al*., 1997).

$CD34^+$ hematopoietic progenitor cell preparation

Bone marrow aspirates were obtained from healthy adult controls. Cord blood was obtained in accord with Institutional Review Board (IRB) guidelines, from placentas discarded after delivery. Mononuclear cells were obtained from the sources described above by density gradient centrifugation using Histopaque (Sigma, St. Louis, MO). Bone marrow (BM) mononuclear cells and umbilical cord blood (UCB) mononuclear cells were resuspended at a density of $0.5–1.5 \times 10^6$ cells/ml in fully supplemented Iscove's modified Dulbecco's medium (IMDM; GIBCO-BRL, Gaithersburg, MD) containing penicillin (100 U/ml), streptomycin (100 μg/ml) (Sigma), and 10% heat-inactivated bovine fetal calf serum (GIBCO-BRL) and then cultured overnight at 37°C/5% CO_2 in a humidified atmosphere. $CD34^+$ cells were then isolated from the nonadherent cell fraction with a MiniMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion of contaminating $CD2^+$ cells was performed using anti-CD2-coated magnetic beads (Dynal, Lake Success, NY). Purified $CD34^+$ cells were also prepared by sorting, using a FACS Vantage cell sorter (Becton Dickinson, San Jose, CA) after staining with fluorescently conjugated anti-CD34 and anti-CD2 (Becton Dickinson). Flow cytometric analysis was ultimately performed on purified

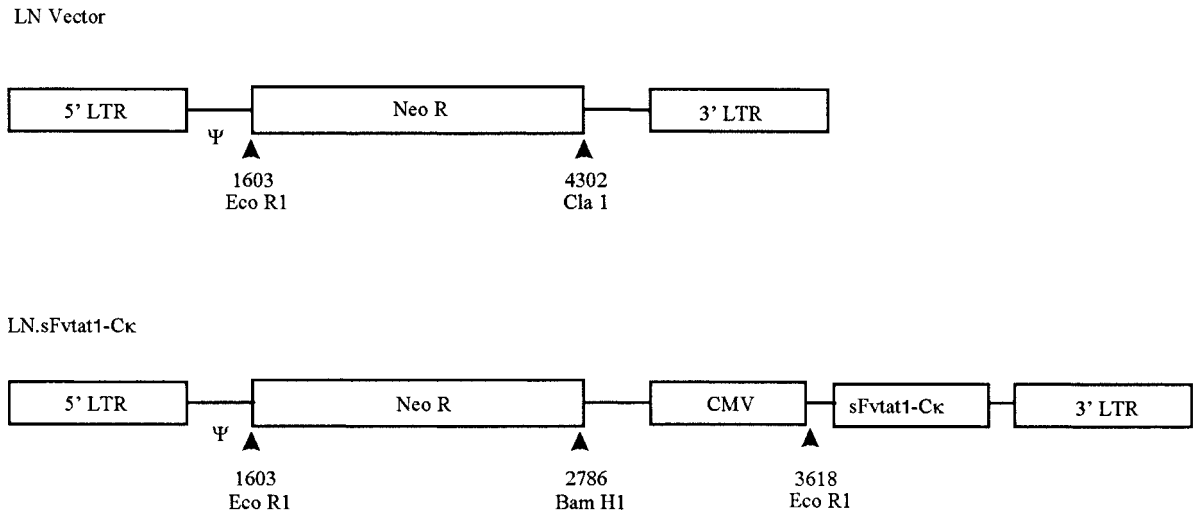

FIG. 1. LN-based vectors. The two LN-based vectors used in this study, LN and LN.sFvtat1-C_κ, are shown. The intracellular antibody gene expression is driven by the CMV promoter in the construct. Each vector contains the neomycin resistance gene. Numbers below each construct relate to cloning sites on the original LN vector from which the vector LN.sFvtat1-C_κ, utilized in this study, was derived (Miller, 1992).

$CD34^+$ cells, using a FACScalibur cytometer (Becton Dickinson). Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (Becton Dickinson) and phycoerythrin (PE)-conjugated anti-CD3 and anti-CD14 were utilized to analyze the purity of $CD34^+$ cells and the abundance of contaminating T cells and monocytes in the selected $CD34^+$ peripheral blood mononuclear cell (PBMC) subpopulation.

Thymic stromal culture and transduction of $CD34^+$ HPCs

Human fetal thymi were obtained from 16- to 22-week abortuses in accord with IRB guidelines. Thymi were disaggregated with surgical scissors in order to produce a cell suspension that also contained fragments of thymic tissue less than 0.5 mm^3 in size. The cell suspension containing thymic fragments was plated into a 24-well plate at 5×10^6 cells per well in fully supplemented IMDM. The medium in thymic cultures was changed initially 48 hr after the establishment of the culture and at 3-day intervals thereafter. On average 60% confluent thymic stromal monolayers were established between 10 and 14 days, at which point the monolayers were irradiated with 35 Gy in preparation for coculture with HPCs.

HPCs derived from adult human BM and UCB were initially cultured at a density of 5×10^3 cells/ml on thymic stromal monolayers for 24 hr in fully supplemented IMDM with interleukin 12 (IL-12, 10 μg/ml) and Flt-3 ligand (10 μg/ml). After overnight culture, HPCs were exposed to vector containing supernatants over four sequential 24-hr time periods. On each occasion HPCs were exposed to a 50:50 mix of vector containing supernatant and supplemented IMDM with added cytokines and continued in coculture with the thymic stroma. After each round of transduction, cells were washed and resuspended in a fresh mix of vector containing supernatant and complete medium containing cytokines. After the four rounds of transduction, cells were cultured in fully supplemented IMDM and cultured at a density of 5×10^3 cells/ml over a monolayer of thymic stroma prepared above. Cocultures of UCB HPCs and thymic stroma were continued in IMDM supplemented with IL-12 and Flt-3 for 21 days. To promote T cell expansion, nonadherent cells were removed from cocultures at 14 days and continued in culture in the presence of IL-2 (60 IU/ml) and phytohemagglutinin (PHA, 10 IU/ml) for a further 7 days.

Assays for transduction efficiency and cell viability were performed on the cultures at 7 and 30 days posttransduction. Nonadherent cells (10^6) were removed from the coculture and transduction efficiencies were determined by semiquantitative polymerase chain reaction (PCR). Transduced and untransduced cells were lysed on day 30 posttransduction and DNA samples were prepared for PCRs as previously described (Poznansky *et al.*, 1991). Amplification of a sequence within the neomycin resistance transgene was performed to determine transduction efficiency, using previously defined primers (Carter *et al.*, 1992). Simultaneous amplification of a human HLA-DQα sequence, using the oligonucleotides GH26/27, was also performed as a DNA control (Shaunak *et al.*, 1990). The frequency of transduced T cell and myelomonocytic populations after challenge with HIV was also established on day 14 postchallenge with HIV-1.

Immunophenotyping and FACS for $CD4^+$ T cells and monocytes generated from transduced $CD34^+$ HPCs

Immunophenotyping of cells generated in the thymic culture system was performed at the time of establishment of the coculture of thymic stroma with HPCs and at 7, 14, and 28 days thereafter. The proportions of cells harvested from cultures were stained with anti-CD3, -CD4, -CD8, -CD14, -CD33, and -CD34. Cells generated in the coculture were then analyzed with a FACScalibur flow cytometer (Becton Dickinson). Cells that were mock transduced or transduced with the LN and LN.sFvtat1-C_κ vectors were also stained with a polyclonal antibody raised against aminoglycoside aminotransferase II (APT-

II). Biotinylated polyclonal antibody to APTII (5 Prime → 3 Prime, Boulder, CO) was added to cells and incubated for 30 min at 4°C. Cells were washed with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS), resuspended in PBS with 1% FCS, and incubated for 30 min at 4°C with streptavidin–FITC or –PE (Becton Dickinson). Cells were then washed with PBS containing 1% FCS and fixed with 1% paraformaldehyde. Flow cytometric analysis was performed within 24 hr, using a dual-laser FACSCalibur (Becton Dickinson Immunocytometry Systems) calibrated with 2-μm Calibrite beads (Becton Dickinson). Data acquisition and analysis were performed with CellQuest software (Becton Dickinson). These transduced and mock-transduced cells were analyzed on the FACScalibur flow cytometer in order to gain an indication of transduction efficiency and in order to establish that the neomycin resistance transgene encoded in the LN and LN.sFvtat1-C_κ vectors was expressed in T cells and monocytes differentiated from transduced $CD34^+$ HPCs.

Reverse transcriptase-polymerase chain reaction analysis of sFvtat1-C_κ expression

mRNA was extracted from 5, 50, 500, 5000, and 50,000 FACS-sorted $CD4^+$ T cells and $CD14^+$ generated from transduced $CD34^+$ cells. RNA extraction was performed with guanidinium thiocyanate and oligo(dT) columns (Pharmacia, Piscataway, NJ). cDNA was prepared by reverse transcription with random primers and Moloney murine leukemia virus reverse transcriptase (GIBCO, Grand Island, NY). cDNA was amplified with primers specific for a 143-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a 410-bp fragment in the sFvtat1-C_κ coding sequence. Primer sequences were as follows: GAPDH (5′ primer, GTGGACCTCATGGCCTACAT; 3′ primer, TGTGAGGGAGATGCTCAGTG); tat2-Cκ (5′ primer, TGGGTTCGCCAGCCTCCAGGA; 3′ primer, ATGTAAATAGGTGATTCCATT). One-quarter of each cDNA product was added to each PCR, with a 1 μM concentration of each oligonucleotide primer and 2.5 U of *Taq* DNA polymerase (Pharmacia). PCR amplification was performed on a GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk CT), for 35 cycles of denaturation at 95°C, annealing at 68°C, and extension at 72°C. PCR products were separated on 1.5% agarose gels.

HIV-1 stocks, HIV-1 challenge, and monitoring of HIV replication and cell viability

Stocks of the HIV-1_{GRF} isolate were established and titered on expanding cultures of PBMCs. Stocks of HIV_{IIIB} laboratory isolate were established and titered on log-phase cultures of the H9 $CD4^+$ T cell line.

Two assay systems were utilized for monitoring the inhibitory action of the transduced antiviral constructs on HIV replication in sorted $CD34^+$ T cells and $CD14^+$ monocytes derived from transduced $CD34^+$ HPCs. Sorted populations of monocytes and T cells generated from HPCs were challenged with the monocytotropic HIV-$1_{JR\text{-}CSF}$ isolate or the T cell-tropic HIV_{IIIB} isolate at a multiplicity of infection of 0.1. Samples of culture supernatant were removed from cultures 3, 6, 9, 12, and 15 days after HIV challenge for HIV-1 p24 antigen estimation by ELISA (Coulter, Miami, FL). Cell viability was also determined after challenge of monocytes and T cells with HIV-1, using trypan blue exclusion.

Statistical analysis

The Student *t* test was used to compare numerical data.

RESULTS

Donor material and $CD34^+$ HPC purity on separation

Bone marrow HPCs were obtained from adult human donors ($n = 6$) and UCB was obtained from discarded placentas ($n = 6$). Purity of $CD34^+$ cells prepared as described above and assessed by immunophenotyping of cells after the separation process varied between 90 and 98%. Purified CD2-depleted $CD34^+$ cells did not contain detectable contaminating T cells as demonstrated by flow cytometry.

TABLE 1. IMMUNOPHENOTYPE OF CELLS GENERATED FROM TRANSDUCED BM AND UCB HPCs[a]

	UCB $CD34^+$ cells			*BM $CD34^+$ cells*		
Immunophenotype	*Mock transduced (%)*	*LN transduced (%)*	*LN.sFvtat1-C_κ transduced (%)*	*Mock transduced (%)*	*LN transduced (%)*	*LN.sFvtat1-C_κ transduced (%)*
$CD3^+CD4^+$	4.2 ± 0.8[b]	2.2 ± 1.1	2.8 ± 0.7	27.8 ± 2.5	21.4 ± 2.8	20.3 ± 2.7
$CD3^+CD8^+$	2.8 ± 0.2	2.1 ± 0.8	1.9 ± 0.3	8.6 ± 0.9	7.0 ± 1.8	7.9 ± 1.1
$CD33^+CD14^+$	86.8 ± 2.8	92.1 ± 6.1	90.1 ± 4.9	59.3 ± 4.9	68.1 ± 4.2	66.4 ± 6.0
Total no. viable cells[c]:	630,000	590,000	490,000	710,000	630,000	610,000

[a]HPCs were mock transduced with heat-inactivated LN vector-containing supernatants or transduced with supernatant containing the LN or LNsFv.tat1-C_κ vector. HPCs were grown in coculture with thymic stroma monolayers in the presence of cytokines as described. The immunophenotype of cells was determined by staining cells with anti-CD3, -CD4, and -CD8 as markers for mature T cells and with anti-CD33 and -CD14 as markers for monocyte-macrophages.

[b]Mean percentage ± standard error; results of three experiments.

[c]Cells generated from 15,000 $CD34^+$ HPCs.

TABLE 2. TRANSDUCTION EFFICIENCIES OF CD34+ HPCs, T CELLS, AND MONOCYTES[a]

	Efficiency of transduction (%)				
		LN		LN.sFvtat1-C_κ	
Day	Mock transduced	No HIV challenge	HIV challenge on day 14	No HIV challenge	HIV challenge on day 14
0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
7	0 ± 0.0	18.5 ± 2.5	18.5 ± 5.0	20 ± 5.0	20 ± 5.0
14	0 ± 0.0	22.5 ± 5.0	22.5 ± 5.0	20 ± 5.0	20 ± 5.0
28	0 ± 0.0	22.5 ± 5.0	17.5 ± 2.5	22.5 ± 2.5	40 ± 7.5

[a]Transduction efficiency was measured in CD34+ HPCs, T cells, and monocytes derived from CD34+ cells 0, 7, 14, and 28 days posttransduction for mock-transduced and LN and LNsFvtat1-C_κ vector-transduced cells, using semiquantitative DNA PCR to detect the neomycin resistance gene sequence. Mock-transduced CD34+ cells were exposed to heat-inactivated LN vector. The transduction efficiency in T cells and monocytes challenged by HIV-1 was also determined 14 days postchallenge with HIV-1_{JR-CSF} and HIV-1_{IIIB}, which was equivalent to 28 days posttransduction. The median and range of results of three separate experiments are shown.

Characterization of the growth potential of transduced HPCs

Adult BM and UCB HPCs were transduced with the vectors described above and subsequently grown in coculture with human fetal thymic stroma. HPCs were grown under conditions that predominantly generated myelo/monocytic cells or that generated both myelomonocytic cells and T cells. Transduced CD34+ HPCs did not generate T cells in the absence of the cytokines IL-12 and Flt-3. Transduced HPCs derived from BM or UCB and cultured to generate a preponderance of myelomonocytes bearing the CD14 and CD33 markers produced similar absolute numbers and percentages of these cells on day 28 whether exposed to transgene-containing or heat-inactivated control vector (Table 1). Transduced HPCs from bone marrow, although not from UCB cultured under conditions favoring T cell development, generated between 6 and 22% $CD3^+CD4^+$ and $CD3^+CD8^+$ cells identifiable immunophenotypically as T cells and underwent expansion in the presence of IL-2 and PHA. Mock-transduced BM HPCs generated sim-

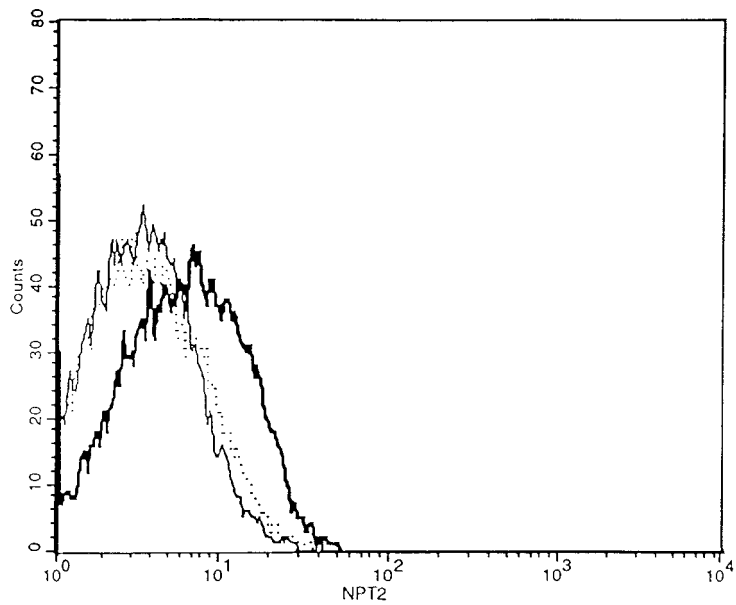

FIG. 2. Distribution of expression of aminoglycoside phosphotransferase transgene (NPT2) encoded in the LN.sFvtat1-C_κ vector. T cells generated from HPCs transduced with LN.sFvtat1-C_κ vector (—) (median fluorescence intensity, 6.21), HPCs mock transduced with heat-inactivated LN.sFvtat1-C_κ vector (· · ·) (median fluorescence intensity, 3.49), or untransduced T cells (—) (median fluorescence intensity, 3.58), were examined by flow cytometry for expression of the neomycin resistance or aminoglycoside transferase transgene product with a polyclonal antibody anti-NPT-2. The results of single representative experiment are shown. Between 14 and 25% of T cells generated from HPCs transduced with the LN.sFvtat1-C_κ vector stained positively for neomycin resistance gene product. CD34+ HPCs transduced with the heat-inactivated LN.sFvtat1-C_κ vector or control untransduced peripheral blood T cells showed no evidence of endogenous NPT-2 activity.

ilar numbers and proportions of single positive T cells that proliferated in response to IL-2 and PHA compared with cells exposed to the sFvtat1-C_κ transgene-containing vector (data not shown).

Efficiency of transduction of HPCs and generated T cells and myelomonocytic cells

Transduction efficiencies of human BM and UCB HPCs were determined by semiquantitative DNA PCR for the neomycin resistance gene or the human HLA-DQα gene as a control for DNA quality. Transduction efficiencies were determined in transduced $CD34^+$ cell populations and pooled T cell and myelomonocytic populations generated from $CD34^+$ cells at days 0, 7, 14, and 28 of culture (Table 2). Transduction efficiencies varied between 18.5 and 22.5% for both LN and LN.sFvtat1-C_κ vectors. The frequency of transduced T cell and myelomonocytic populations after challenge with HIV was also assayed on day 14 postchallenge with HIV. There was a significant rise in the proportion of transduced cells in cultures challenged with HIV, from 20.0 to 40% ($p < 0.05$, Student t test) (Table 2). Expression of the neomycin resistance transgene, aminophosphotransferase II (APT-II), was also demonstrated by flow cytometry in monocytes and T cells differentiated from $CD34^+$ cells that had previously been transduced with the LN or LN.sFvtat1-C_κ vector. Transduction efficiencies for the two vectors varied between 14 and 25% as determined by flow cytometry (Fig. 2). Labeling of T cells derived from mock-transduced $CD34^+$ cells or untreated peripheral blood T cells demonstrated no endogenous neomycin resistance gene expression (Fig. 2).

RT-PCR for expression of tat2-Ck transgene

RT-PCR was performed on T cells and monocytes generated from $CD34^+$ cells transduced with the LN or LN.sFvtat1-C_κ vector (Fig. 3A and B). The RT-PCR on cDNA from 50, 500, 5000, and 50,000 sorted T cells generated from LN.sFvtat1-C_κ-transduced $CD34^+$ cells generated a 410-bp band with the primary amplifying cDNA encoding the sFvtat1-C_κ product. This band was intermittently generated by RT-PCR cDNA prepared from five T cells. The control of 143-bp product generated from GAPDH cDNA was present in RT-PCR performed on equivalent numbers of transduced T cells. Amplification of cDNA from sorted monocytes generated from $CD34^+$ cells transduced with the LN.sFvtat1-C_κ vector generated similar results (data not shown). A total of 50,000 T cells generated from $CD34^+$ cells transduced with the LN vector did not generate the 410-bp band representing sFvtat1-C_κ product (Fig. 3A). Negative controls containing no cDNA did not generate a product with the GAPDH primers (Fig. 3B).

Protection of lymphocytes from HIV infection

Sorted $CD3^+CD4^+$ T cells and $CD14^+$ monocytic cells generated from transduced and mock-transduced HPCs were challenged with HIV. These HIV-challenged cells were maintained in culture in the absence of neomycin selection and HIV p24 assays were performed on culture supernatants 0, 3, 6, 9, 12, and 15 days after HIV challenge. $CD3^+CD4^+$ T cells produced significantly lower amounts of HIV-1 p24 when generated from

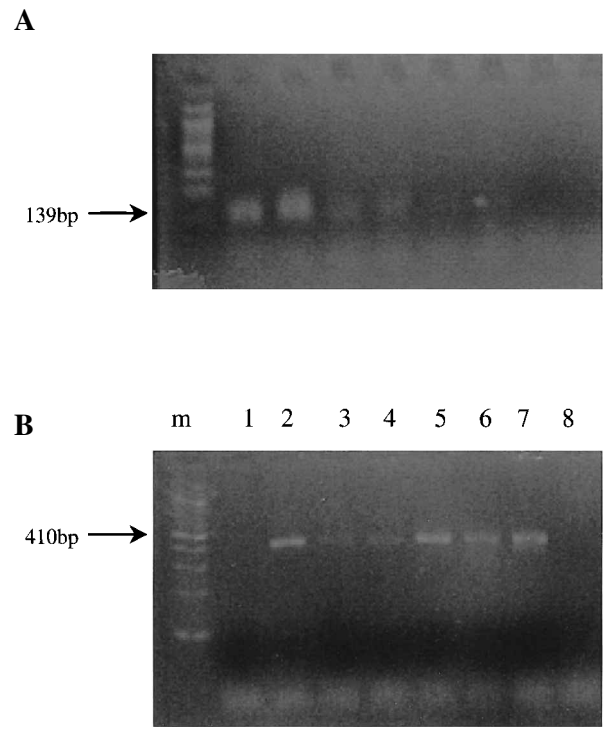

FIG. 3. RT-PCR detects sFvtat1-C_κ RNA in $CD14^+$ monocytes and $CD3^+CD4^+$ T cells generated from $CD34^+$ cells transduced with the LN.sFvtat1-C_κ retroviral vector. cDNA was synthesized from mRNA derived from sorted $CD3^+CD4^+$ T cells generated from $CD34^+$ cells transduced with the LN or LN.sFvtat1-C_κ vector. Primers were used to detect the presence of GAPDH and sFvtat1-C_κ cDNA derived from mRNA was extracted from 5 to 50,000 FACS-sorted $CD4^+$ T cells. PCR products were separated on 1.5% agarose gels. The control 143-bp product generated from GAPDH cDNA was present in RT-PCR performed on 50,000 (lane 1), 5000 (lane 2), 500 (lane 3), 50 (lane 4), and 5 cells (lanes 5–7). Negative controls containing no cDNA did not generate a product with the GAPDH primers (**A,** lane 8). RT-PCR performed on sorted T cells generated from LN.sFvtat1-C_κ transduced $CD34^+$ cells generated a clear 410-bp band, with the primers amplifying cDNA encoding the sFvtat1-C_κ product intermittently from 5 cells (lanes 1–3) and from 50 cells (lane 4), 500 cells (lane 5), 5000 cells (lane 6), and 50,000 cells (lane 7) **(B).** A total of 50,000 T cells generated from $CD34^+$ cells transduced with the LN vector did not generate the 410-bp band representing sFvtat1-C_κ product (**B,** lane 8). Amplification of cDNA from sorted $CD14^+$ monocytes generated from $CD34^+$ cells transduced with the LN.sFvtat1-C_κ vector generated similar results (data not shown).

$CD34^+$ cells transduced with the LNsFvtat1-C_κ vector as compared with cells transduced with the LN vector or mock-transduced cells ($p < 0.005$; Student t test) (Fig. 4A). Peak HIV-1 p24 production was significantly reduced ($p < 0.001$; Student t test) in sorted populations of $CD14^+$ monocytes generated from BM $CD34^+$ HPCs that had been transduced with the LNsFvtat1-C_κ vector as compared with cells transduced with the LN vector or mock-transduced cells (Fig. 4B).

The viability of T cells (Fig. 5A) and monocytic cells (Fig. 5B) was also determined in the cultures after challenge with

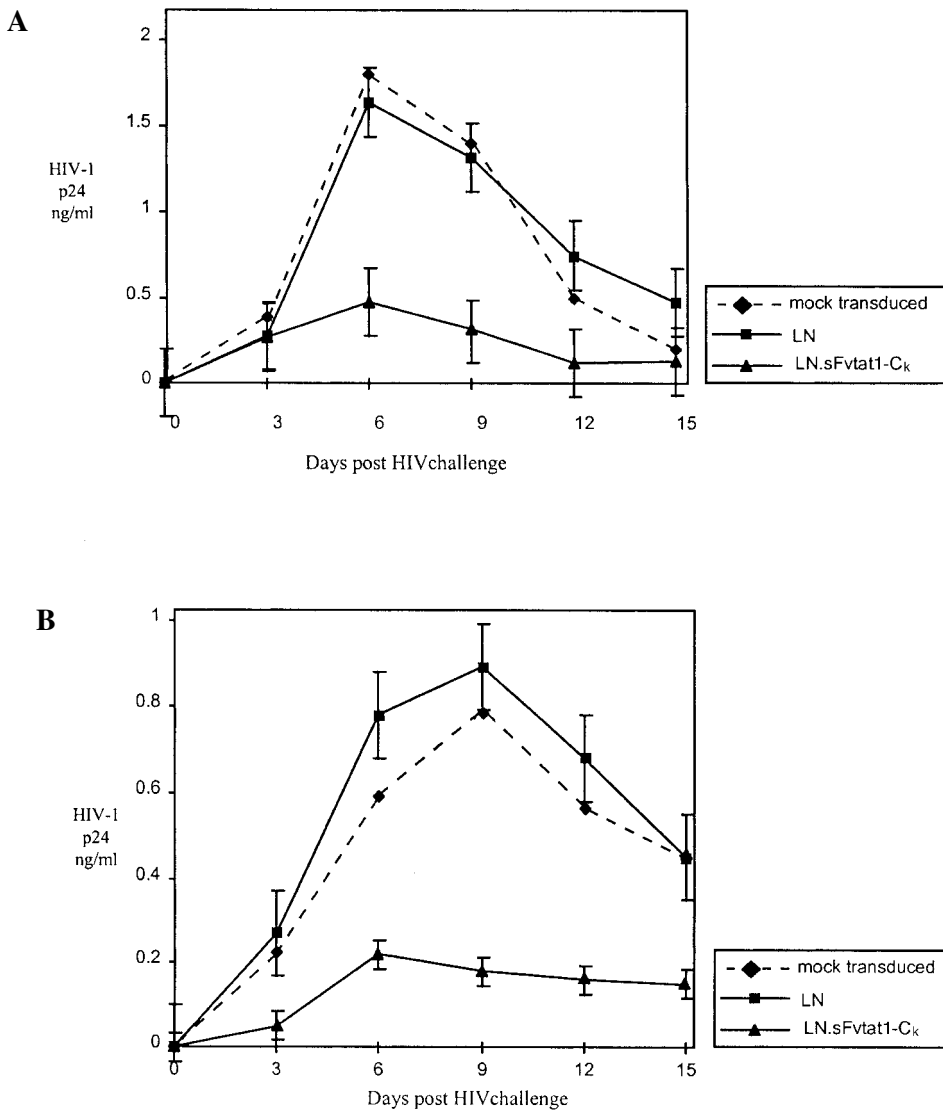

FIG. 4. HIV-1 replication was assessed in pooled populations of monocytes or T cells challenged with HIV-1_{JR-CSF} **(A)** or HIV-1_{IIIB} **(B),** respectively. Monocytes and/or T cells were generated from UCB or adult BM-derived $CD34^+$ HPCs were transduced with the LN.sFvtat1-C_κ vector (▲) or the LN (■) vector or underwent a mock transduction process involving the exposure of HPCs to supernatants from PG13 cells (◆). HIV-1 replication was monitored in cultures at regular intervals (days 3, 6, 9, and 14) postchallenge with HIV-1. Standard errors of the means are shown for the cells transduced with the LN and LN.sFvtat1-C_κ vectors. Data presented represent the means and SEM from three independent experiments performed in triplicate.

HIV-1. Cells generated from $CD34^+$ HPCs transduced with the LN.sFvtat1-C_κ vector demonstrated higher numbers of viable cells 15 days after HIV challenge ($p < 0.05$; Student t test) as compared with cells generated from LN-transduced HPCs. The highest viability was seen in sorted T cells and monocytes generated from $CD34^+$ cells and exposed to heat-inactivated HIV-1 alone.

DISCUSSION

This study demonstrates that both pooled and sorted populations of T cells and monocytes generated from UCB- and BM-derived HPCs transduced with an intracellular and antibody gene targeting HIV-1 Tat are protected from HIV replication. Previous studies have shown that a number of antiviral constructs including intracellular antibodies directed against Tat and gp120, ribozymes targeting the CAP site of HIV RNA, dominant negative proteins targeting the function of HIV Rev, and decoy RNA molecules inhibiting the transcriptional control induced by HIV Tat or Rev can inhibit HIV replication in transduced primary $CD4^+$ cells to varying degrees (Malim *et al.*, 1992; Marasco *et al.*, 1993; Dropulic and Jeang, 1994; Poznansky *et al.*, 1998b; Mhashilkar *et al.*, 1999). Several phase 1 clinical trials have been performed using the transduction and *ex vivo* expansion of T cells transduced with the anti-HIV construct (Haubrich *et al.*, 1995; Leavitt *et al.*, 1996; Morgan and Walker, 1996; Ranga *et al.*, 1998). These trials have demonstrated the safety of this ap-

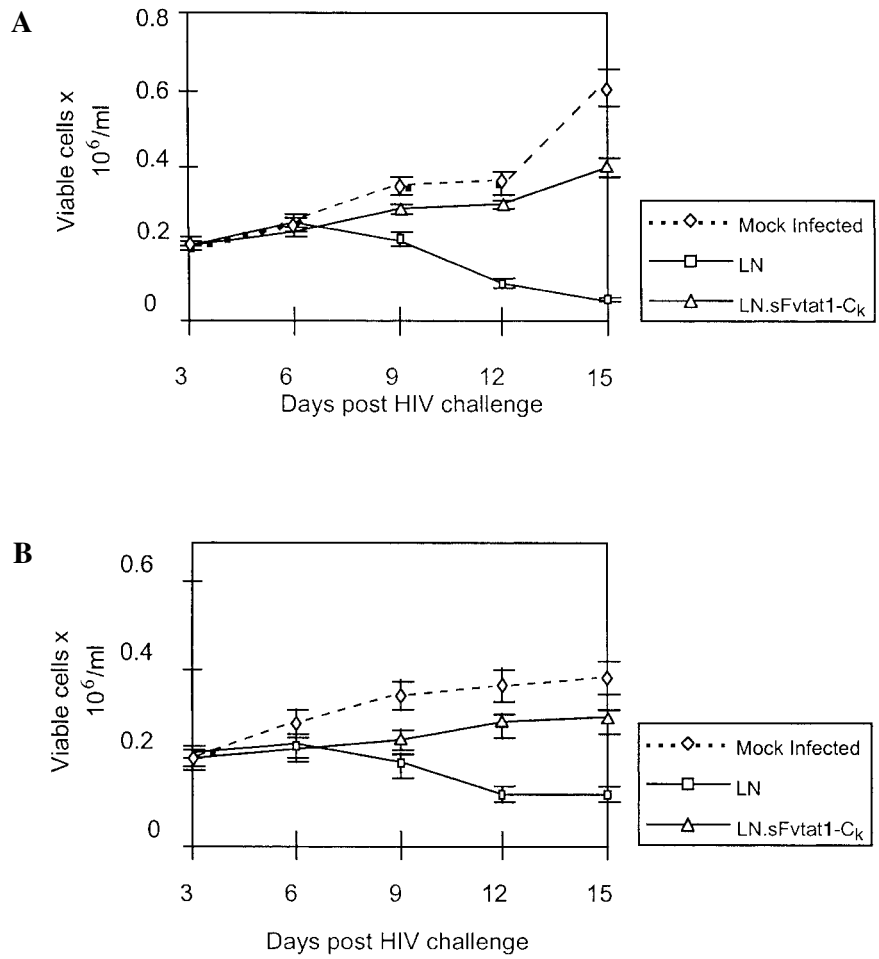

FIG. 5. The viability of T cells **(A)** and monocytes **(B)** generated from transduced $CD34^+$ cells and challenged with HIV-$1_{JR\text{-}CSF}$ and HIV-1_{IIIB} was determined. The number of viable cells in expanding cell cultures of T cells and monocytes derived from transduced $CD34^+$ cells was assessed at intervals up to 15 days after challenge with HIV-1. $CD34^+$ cells were transduced with the construct LN (□) or LN.sFvtat1-C_κ (△). Cells derived from transduced $CD34^+$ cells were also mock infected with heat-inactivated HIV-1 (◇). Data presented are the means and SEM from three independent experiments.

proach and the most recent trial by Nabel *et al.* has shown efficacy with regard to the influence of the antiviral construct on enhanced T cell engraftment *in vivo* (Ranga *et al.*, 1998). The drawback of using a target population of mature T cells is that, whatever change is effected in them, the life span of these cells is highly variable; many are relatively short-lived cells and the transduced component will therefore ultimately make up only a small proportion of the entire circulating T cell population (Poznansky *et al.*, 1998b). Furthermore, a gene therapy protecting only mature T cells does not address the HIV replication that occurs in hematopoietic cells other than T cells, such as dendritic cells and monocyte-macrophages, in which persistent and long-lived HIV replication has been clearly demonstrated. As a consequence of these drawbacks to current approaches targeting mature T cells, a stem/progenitor cell gene therapy has been proposed in order to create a potentially more durable population of HPCs capable of reconstituting individuals with multiple generations of cells capable of differentiating along various hematopoietic lineages including T cells, monocyte-macrophages, and dendritic cells.

We have previously demonstrated that T cells generated from BM HPCs in cocultures with human fetal thymic stroma have the characteristics of mature single positive T cells; evidence includes rearrangement of the T cell receptor β-chain gene by PCR, RAG gene expression by RT-PCR, IL-2 generation by ELISA, and proliferation in response to activation by mitogens (Freedman *et al.*, 1995; Rosenzweig *et al.*, 1997). This study further adds the finding that HPCs cultured in this way are effectively transducible at efficiencies between 20 and 28% with LN-based murine retroviral vectors and that the transduction process does not significantly alter the proportions of differentiated cells of T cell or monocyte-macrophage immunophenotype generated in the *in vitro* system. There was a slight decrease in the number of differentiated cells generated in the coculture system as compared with HPCs that were exposed to heat-inactivated LN vector-containing supernatants, but this did not reach significance (Table 2).

$CD3^+CD4^+$ T cells generated from $CD34^+$ cells transduced with the LNsFvtat1-C_κ vector expressed the sFvtat1-C_κ transgene as determined by RT-PCR and proliferated in response to

IL-2. Similarly, immunophenotypically normal $CD4^+CD14^+$ monocytes generated from LNsFvtat1-C_κ-transduced $CD34^+$ cells expressed the sFvtat1-C_κ transgene as determined by RT-PCR.

Intracellular antibodies to both Tat and gp120 have now been shown to be effective in inhibiting HIV replication in various *in vitro* systems, and have been shown to be active against both laboratory and primary isolates of HIV (Marasco *et al*., 1993; Mhashilkar *et al*., 1995; Poznansky *et al*., 1998b). This study demonstrates the efficacy of the anti-Tat intracellular antibody in inhibiting HIV replication in cells maturing along T cell and monocyte lineages in the absence of selection for the transduced HPCs or their offspring. Further data presented here demonstrate that transduction efficiency increases from 22.5 to 40% in T cells and monocytes after HIV challenge, and the viability of cultures of T cells and monocytes generated from $CD34^+$ HPCs after HIV challenge was greater if the HPCs were transduced with the LN.sFvtat1-C_κ construct as compared with those cells derived from the HPCs transduced with the LN vector.

Protection against HIV replication occurs in this system despite a mean transduction efficiency of 25%. This may have been due in part to the selective expansion of cells generated from $CD34^+$ HPCs transduced with LN.sFvtat1-C_κ, as evidenced by the increase in transduced T cells in HIV-challenged cultures. Alternatively, the sFvtat1-C_κ transgene product may be active in *trans* between transduced and untransduced cells.

The aim of a gene therapy for HIV infection is to reconstitute the infected individual with long-lasting and potentially self-regenerating populations of $CD4^+$ T cells, monocyte-macrophages, and dendritic cells, protected from HIV infection and/or HIV replication, *in vivo*. This is theoretically possible if the antiviral construct is introduced into the $CD4^+$ progenitor cells or hematopoietic stem cells (Yu *et al*., 1994) and if the transgene is expressed and protects cells during the maturation process. The data presented here indicate that transduction of HPCs can occur with reasonable efficiency, without perturbing the differentiation capacity of progenitor cells, and can be expressed during T lymphoid and monocytic maturation. Furthermore, this study demonstrates the proof of principle *in vitro* that the introduction of a gene encoding the anti-HIV intracellular antibody against Tat into HPCs from BM or UCB gives rise to T cells and monocyte-macrophages that are protected from HIV and have a selective growth advantage. This study paves the way for testing the principle of a progenitor cell gene therapy for HIV infection within an animal model such as the SCID-Hu mouse and ultimately in a phase 1 trial in human HIV-infected volunteers.

REFERENCES

BELMONT, J.W., MACGREGOR, G.R., WAGNER-SMITH, K., FLETCHER, F.A., MOORE, K.A., HAWKINS, D., VILLALON, D., CHANG, S.M.W., and CASKEY, C.T. (1988). Expression of human adenosine deaminase in murine hematopoietic cells. Mol. Cell. Biol. **8,** 5116.

BRENNER, M.K., CUNNINGHAM, J.M., SORRENTINO, B.P., and HESLOP, H.E. (1995). Gene transfer into human hematopoietic progenitor cells. Br. Med. Bull. **51,** 167–191.

CARTER, R.F., ABRAMS-OGG, A.G., DICK, J.E., KRUTH, S.A., VALLI, W.E., KAMELREID, S., and DUBE, I.D. (1992). Autologous transplantation of canine long-term marrow culture cells genetically marked by retroviral vectors. Blood **79,** 356.

DROPULIC, B., and JEANG, K.-T. (1994). Gene therapy for human immunodeficiency virus infection: Genetic antiviral strategies and targets for intervention. Hum. Gene Ther. **5,** 927–939.

DUNBAR, C.E. (1996). Gene transfer to hematopoietic stem cells: Implications for gene therapy of human disease. Annu. Rev. Med. **47,** 11–20.

FREEDMAN, A.R., ZHU, H., LEVINE, J.D., KALAMS, S., and SCADDEN, D.T. (1995). Generation of human T-lymphocytes from bone marrow CD34+ cells in vitro. Nature Med. **2,** 46–51.

HAUBRICH, R., MCCUTCHAN, J.A., HOLDREDGE, R., HEINER, L., MERRITT, J., MERCHANT, E. (1995). An open label, phase I/II clinical trial to evaluate the safety and biological activity of HIV-IT (V) (HIV-1IIIBenv/rev retroviral vector) in HIV-1-infected subjects. Hum. Gene Ther. **6,** 941–955.

KARLSSON, S. (1991). Treatment of genetic defects in hematopoietic cell function by gene transfer. Blood **78,** 2481–2492.

KOTANI, H., NEWTON, P.B., ZHANG, S., CHIANG, Y.L., OTTO, E., WEAVER, L., BLAESE, M., FRENCH ANDERSON, W., and MCGARRITY, G.J. (1994). Improved methods of retroviral vector transduction and production for gene therapy. Hum. Gene Ther. **5,** 19–28.

LEAVITT, M.C., YU, M., WONG-STAAL, F., LOONEY, D. (1996). Ex vivo transduction and expansion of $CD4^+$ lymphocytes from HIV^+ donors: Prelude to a ribozyme gene therapy trial. Gene Ther. **3**(7), 599–606.

LU, M., MARUYAMA, M., ZHANG, N., LEVINE, F., FRIEDMANN, T., and HO, A. (1994). High efficiency retroviral-mediated gene transduction into $CD34^+$ cells purified from peripheral blood of breast cancer patients primed with chemotherapy and GM-CSF. Hum. Gene Ther. **5,** 203–208.

MALIM, M.H., FREIMUTH, J., LIU, J., BOYLE, T.J., LYERLY, H.K., and CULLEN, B.R. (1992). Stable expression of transdominant Rev protein in human T-cells inhibits HIV replication. J. Exp. Med. **176,** 1197–1201.

MARASCO, W.A., HASELTINE, W.A., and CHEN, S.-Y. (1993). Design, intracellular expression and activity of a human anti-human immunodeficiency virus type 1 gp120 single chain antibody. Proc. Natl. Acad. Sci. U.S.A. **90,** 7889–7893.

MARASCO, W.A., CHEN, S.Y., RICHARDSON, J., RAMSTEDT, U., and JONES, S. (1998). Clinical protocol: Intracellular antibody against HIV-1 envelope protein for AIDS gene therapy. Hum. Gene Ther. **9,** 1627–1642.

MHASHILKAR, A.M., BAGLEY, J., CHEN, S.Y., SZILVAY, A.M., HELLAND, D.G., and MARASCO, W.A. (1995). Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by anti-Tat single chain intrabodies. EMBO J. **14,** 1542–1551.

MHASHILKAR, A.M., LA VECCHIO, J., EDEHARDT, B., BROOKS, J., BOISOT, S., DOVE, J.H., PUMPHREY, C., LI, X., WEISSMAHR, R.N., RING, D.B., RAMSTEDT, U., and MARASCO, W.A. (1999). Inhibition of human immunodeficiency virus type 1 replication in vitro in acutely and persistently infected human CD4+ mononuclear cells expressing murine and humanized anti-HIV-1 Tat sFv intrabodies. Hum. Gene Ther. **10,** 1453–1467.

MILLER, A.D. (1992). Retroviral vectors. Curr. Top. Microbiol. Immunol. **158,** 1–24.

MORGAN, R.A. and WALKER, R. (1996). Gene therapy for AIDS using retroviral mediated gene transfer to deliver HIV-1 antisense TAR and transdominant Rev protein genes to syngeneic lymphocytes in HIV-1 infected identical twins. Hum. Gene Ther. **7,** 1281–1306.

MORITZ, T., KELLER, D.C., and WILLIAMS, D.A. (1993). Human cord blood cells as targets for gene transfer: Potential use in gene therapies of severe combined immunodeficiency disease. J. Exp. Med. **178,** 529–536.

NABEL, G.J., FOX, B.A., POST, L., THOMPSON, C.B., and WOFFENDIN, C. (1994). Clinical protocol: A molecular genetic intervention for AIDS—effect of a transdominant negative form of *rev*. Hum. Gene Ther. **5,** 79–92.

POZNANSKY, M.C., WALKER, B., HASELTINE, W.A., SODROSKI, J., and LANGHOFF, E. (1991). A rapid method for quantitating the frequency of peripheral blood cells containing HIV-1 DNA. J. Acquir. Immune Defic. Syndr. **4,** 368–373.

POZNANSKY, M.C., ADAMS, G.B., and McCLURE, M. (1998a). Gene therapy for the treatment of HIV-1 infection. In *Biotechnology*, Vol. 5. (Wiley-VCH, Verlag, Gmbh, Germany).

POZNANSKY, M.C., FOXALL, R., MHASHILKAR, A., COKER, R., JONES, S., RAMSTEDT, U., and MARASCO, W.A. (1998b). Inhibition of human immunodeficiency virus replication and growth advantage of CD4+ T-cells from HIV-infected individuals that express intracellular antibodies against HIV-1 gp120 or Tat. Hum. Gene Ther. **9,** 487–496.

RANGA, U., WOFFENDIN, C., VERMA, S., XU, L., JUNE, C.H., BISHOP, D.K., and NABEL, G. (1998). Enhanced T cell engraftment after retroviral delivery of an antiviral gene in HIV-infected individuals. Proc. Natl. Acad. Sci. U.S.A. **95**(3), 1201–1206.

ROSENZWEIG, M., MARKS, D.F., ZHU, H., HEMPEL, D., MANSFEILD, K.G., SEHGAL, P.K., KALAMS, S., SCADDEN, D.T., and JOHNSON, R.P. (1996). In vitro T-lymphopoiesis of human and rhesus CD34+ progenitor cells. Blood **87,** 4040–4048.

ROSENZWEIG, M., MARKS, D.F., HEMPEL, D., LISZIEWICZ, X., and JOHNSON, R.P. (1997). Transduction of CD34+ hematopoietic progenitor cells with an anti-*tat* gene protects T-cell and macrophage progeny from AIDS virus infection. J. Virol. **71,** 2749–2746.

SHAUNAK, S., ALBRIGHT, R.E., KLOTMAN, M.E., HENRY, S.C., BARTLETT, J.A., and HAMILTON, J.D. (1990). Amplification of HIV-1 provirus from cerebrospinal fluid and its correlation with neurologic disease. J. Infect. Dis. **161,** 1068–1072.

YAMADA, O., YU, M., YEE, J.-K., KRAUS, G., LOONEY, D., and WONG-STAAL, F. (1994). Intracellular immunization of human T-cells with a hairpin ribozyme against human immunodeficiency virus type 1. Gene Ther. **1,** 38–45.

YU, M., PEOSCHLA, E., and WONG-STAAL, F. (1994). Progress towards gene therapy for HIV infection. Gene Ther. **1,** 13–26.

YU, M., LEAVITT, M.C., MARUYAMA, M., YAMADA, O., YOUNG, D., HO, A.D., and WONG-STAAL, F. (1995). Intracellular immunization of human fetal cord blood stem/progenitor cells with a ribozyme against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U.S.A. **92,** 699–703.

Address reprint requests to:
Dr. Mark Poznansky
Partners AIDS Research Center
Massachusetts General Hospital
13th Street, Bldg. 149, Room 52120
Charlestown, MA 02129-2000

Received for publication December 30, 1998; accepted after revision July 21, 1999.