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Nonviral and viral delivery of a human immunodeficiency virus protective gene into primary human T cells

(gene therapy/AIDS/retroviral vector/particle-mediated gene delivery/Rev)

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ABSTRACT **Because AIDS has been refractory to traditional pharmacologic interventions, alternative approaches have been developed. Although the introduction of specific antiviral genes into T leukemia cells can provide relative resistance to human immunodeficiency virus (HIV) replication, the testing of such genes against primary viral isolates in human CD4+ lymphocytes has been limited, and safety questions remain regarding gene delivery into cells from HIV-infected patients. In this report, we evaluate the efficacy of a transdominant mutant protein, Rev M10, against cloned and primary HIV isolates in human peripheral blood lymphocytes and describe different methods of gene transfer into peripheral blood lymphocytes from HIV-infected individuals. We show that gold microparticles can mediate stable Rev M10 gene transfer into these cells. Introduction of Rev M10 by these techniques conferred resistance to HIV infection *in vitro* to cloned and clinical isolates. Nonviral delivery of HIV protective genes will facilitate the development of gene therapy for AIDS and the analysis of viral and cellular gene expression in human T lymphocytes.**

Replication of the human immunodeficiency virus (HIV) requires interactions among the viral genome, regulatory proteins synthesized by the virus, and cellular factors (1). Of these, Rev is an essential viral protein translated from a highly spliced viral mRNA synthesized early in virus infection. This 19-kDa nuclear protein (2) acts in concert with host cell factors to facilitate the export of unspliced viral mRNAs into the cytoplasm and is thought to be important in regulating virus latency (3, 4). Mutations in its coding sequence greatly reduce viral replication and particle formation (5). Additional mutational analysis of this protein has identified a highly conserved leucine-rich domain of the protein that is required for Rev function, presumably by interacting with host factors. Mutations in this region have given rise to a defective protein that acts as a transdominant inhibitor, which inhibits HIV replication in T leukemia cells and does not affect several normal T-cell functions (6–10).

Although such HIV inhibitory genes are effective in cell culture, the ability of such molecular genetic interventions to affect the treatment of AIDS is unknown, and several important issues remain to be addressed: (*i*) The protective gene must be shown to be effective in the relevant target cells, the peripheral blood lymphocytes (PBL). (*ii*) It would be preferable to deliver such protective genes into cells with nonviral vectors, which minimize the potential to generate replication-competent virus in immunocompromised individuals. (*iii*) It is necessary to stimulate T cells to achieve optimal gene transfer when using viral vectors, and the process of T-cell activation induces virus replication (11, 12). The gene therapy procedure must, therefore, be accomplished without activating latent virus.

In this study, we evaluate the efficacy of Rev M10 in human peripheral blood CD4+ cells. These cells were genetically modified by using retroviral vectors or by particle-mediated gene transfer. We find that both methods allow delivery of Rev M10, which can provide protection against challenge with cloned and primary HIV isolates. The efficacy of particle-mediated gene transfer was comparable to or better than retroviral delivery, showed stable expression and integration, and hence offers potential safety advantages. These findings will facilitate efforts to develop clinically relevant antiviral gene transfer approaches to the treatment of HIV infection.

MATERIALS AND METHODS

Plasmids. A plasmid that contains the Rous sarcoma virus (RSV) promoter and tat-activation response element (TAR) sequence from −18 to +72 of HIV, pRSV/TAR Rev M10, was used to express the Rev M10 open reading frame (10). pRSV/TAR ΔRev M10 is identical to pRSV/TAR Rev M10, except that the initiation codon ATG and a small region of linker was deleted that was confirmed by sequence analysis.

Isolation and Passage of Human PBL. Blood for these studies was obtained from normal or HIV-seropositive donors. PBL were isolated using Ficoll/Hypaque separation (13). The cells were stimulated in flasks coated with immobilized OKT3 monoclonal antibody and soluble interleukin 2 (IL-2; 50 units/ml) for 48–72 hr. Cells were recovered and resuspended at 5×10^5 cells per ml in either AIM-V medium (BRL) or X-Vivo-15 medium (BioWhittaker) containing 5% human AB serum plus IL-2 at 50 units per ml. Cells were maintained at 2×10^5 to 1.5×10^6 cells per ml throughout the experiments.

Retroviral Transduction. Freshly isolated human PBL from different normal HIV-seronegative donors were purified by centrifugation on Ficoll gradients. Cells were then stimulated either by treatment with phytohemagglutinin at 5 μg/ml and IL-2 at 50 units/ml for 48 hr or treatment with immobilized anti-CD3 and IL-2 at 50 units/ml for up to 72 hr. Retroviral vectors were derived from the pLJ plasmid (14). After stimulation, cells were infected for 6–12 hr with ψ-Crip supernatants (plus Polybrene at 5 μg/ml) containing pLJ-Rev M10 neo or ATG-deletion mutant pLJ-ΔRev M10 neo retroviruses (7). Cells were incubated at a density of 1×10^6 cells per well in a 24-well plate containing 1 ml of ψ-Crip super-

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Abbreviations: PBL, peripheral blood lymphocytes; RT, reverse transcriptase; IL-2, interleukin 2; HIV, human immunodeficiency virus; m.o.i., multiplicity of infection.

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natant and 1 ml of AIM-V medium. After infection, cells were washed once by centrifugation and resuspended at 5×10^5 cells per ml in conditioned AIM-V medium plus IL-2 at 50 units/ml. Cells were then selected with G-418 (active) at 300 μg/ml for 7 days.

Particle-Mediated Gene Transfer. Plasmid DNA was linearized by digestion with *Aat* II restriction enzyme, extracted using phenol/chloroform, precipitated with ethanol, and resuspended in Tris/EDTA buffer, pH 8.0, to a final concentration of 1 mg/ml. Microscopic gold particles (60 mg, 1.6 μm in diameter) were washed with 1 ml of 70% (vol/vol) ethanol, mixed for 3–5 min, incubated at room temperature for 2 min, and pelleted by microcentrifugation. The particles were then washed three times in sterile water (by adding water, mixing for 1 min, and allowing particles to settle) and resuspended in 1 ml of 50% (vol/vol) glycerol. Five micrograms of the linearized DNA was added to 3 mg (50 μl) of the gold microcarriers. $CaCl_2$ (final concentration, 1 M) and spermidine (final concentration, 16 mM) were added to the mixture while mixing vigorously. The DNA coated particles were pelleted by centrifugation. Graded ethanol washes were done, and the pellet was resuspended in 50 μl of 100% ethanol. A suspension of 500 μg (8 μl) of microcarriers was removed and spread evenly over the central 1 cm^2 of the Mylar macrocarrier sheet. This suspension was allowed to dry for 10–15 min before the bombardment procedure.

The apparatus used to deliver plasmid DNA was the Biolistic PDS-1000/He System manufactured by Bio-Rad; the apparatus was set up as described by the manufacturer. Particles were delivered by a modification of a reported procedure (15). The following adjustments were made: rupture disk macrocarrier gap, 3/4 inch (1 inch = 2.54 cm); macrocarrier travel distance, 6 mm; chamber vacuum, 15 inches of Hg; helium pressure, 1800 psi (1 psi = 6.9 kPa); and target distance, 4 cm. Immediately before transfection, PBL were centrifuged and resuspended to 5×10^6 cells per 100 μl; the cell suspension was then spread over a 4-cm^2 area of a 35-mm Petri dish. After transfection, cells were quickly removed from the Petri dish and resuspended at 5×10^5 cells per ml in conditioned medium.

HIV Infections. Cells were challenged with either HIV^{BRU} or fresh singly passaged HIV clinical isolates HIV^{CLIN}. Cells (1×10^6 cells per ml) were incubated with HIV at a specific multiplicity of infection (m.o.i.) for 2–4 hr at 37°C. After incubation, cells were centrifuged with a 20× vol of fresh medium and resuspended at 5×10^5 cells per ml in medium without G-418. Cells were maintained at a density of 0.2–1.5 $\times 10^6$ cells per ml throughout the infection.

Reverse Transcriptase (RT) Assays. Culture supernatants were assayed for RT activity as described (16). Poly(A)/oligo(dT) was used as template primer, and incorporation of [^{32}P]dTTP was measured after spotting 5 μl of the RT reaction mixture onto DE81 paper and washing with 2× sodium chloride/sodium citrate (1× solution is 0.15 M sodium chloride/0.017 M sodium citrate) four times. Radioactivity was analyzed on a Betagene (Waltham, MA) Betascope. RT activity is expressed as cpm/ml of culture supernatants. p24 antigen levels (Coulter) correlated with RT levels when compared in selected experiments.

Southern Blotting. Genomic DNA was isolated (17) from PBL transfected with Rev M10 by particle-mediated gene transfer and selected for 2 weeks in G-418 (300 μg/ml). DNA was resuspended in Tris/EDTA buffer, digested with RNase I (40 μg/ml), and then with relevant restriction enzymes (Fig. 3 legend) for 4 hr at 37°C. The digested DNA (10 μg) was precipitated and electrophoresed on a 0.7% agarose gel. DNA was transferred to GeneScreen (NEN) and hybridized with a probe consisting of the entire Rev M10 coding region labeled by oligonucleotide priming (18) according to the manufacturer's recommendations. Conditions for hybridization and transfer were as described (17).

Quantitative PCR Analysis. To analyze gene transfer frequencies, limiting-dilution PCR was done. Briefly, cells carrying Rev M10 or ΔRev M10 were diluted into CEM cells (10) at progressively lower cell numbers. Chromosomal DNA was prepared from a total of 10^5 cells using a quick lysis method. The cell pellet was suspended in 10 mM KCl/1 mM Tris·HCl, pH 8.3/0.25 mM $MgCl_2$ (50 μl) and an equal volume of solution B (1 mM Tris·HCl, pH 8.3/0.25 mM $MgCl_2$/0.1% Tween 20/0.1% Nonidet P-40/proteinase-K at 50 mg/ml). The reaction mixture was incubated at 56°C for 1 hr and at 95°C for 20 min. Cell lysate (5 μl) was used in the PCR reaction without further purification of the DNA to minimize the risk of false positive results. Cell lysate (5 μl) equivalent to 1000, 100, 10, 5, 2, or 0.1 cells with the target DNA in a background of DNA derived from 25,000 CEM cells was analyzed. The PCR mixture contained 50 mM KCl, 10 mM Tris·HCl (pH 8.8), 0.001% gelatin, 125 nM primers, 200 μM dNTPs, 1.5 mM $MgCl_2$, and 0.6 unit of *Taq* polymerase (Promega) in a final 25-μl vol. Amplification was done in a thermal cycler (Perkin–Elmer/Cetus) for a total of 45 cycles, each cycle consisting of melting at 91°C, annealing at 56°C, and extension at 72°C; each step was for 1 min. The amplified products were separated on 1% agarose gel and directly visualized by ethidium bromide staining. The nucleotide sequences for Rev M10 and ΔRev M10 of the primers were chosen to match each vector specifically as follows: sense primers (*i*) for pLJ Rev M10 (9335A) 5′-TCTTGTCTGCCAGATCCCGGATCCAT-3′; (*ii*) for pLJΔRev M10 (3111B) 5′-TTAAGTGACCAGCTACAGTCGGAA-3′; (*iii*) for pRSV/TAR Rev M10 (3112B) 5′-GCTTAAGCTCATGGCAGGAA-3′; (*iv*) for pRSV/TAR Rev M10 (3113B) 5′-GGAACCCAGTGCTTAAGCTTG-3′. A common antisense primer (3060B) 5′-CTCGTTACAATCAAGAGTTCTCAGATC-3′ was used in all reactions.

RESULTS

Protective Effects of Rev M10 in Human PBL with Retroviral Vector Delivery. To determine whether expression of Rev M10 could provide resistance to HIV infection in human lymphocytes, we transduced cells with a murine amphotropic retroviral vector, pLJ Rev M10, which synthesizes biologically active Rev M10 protein (7) or an ATG-deletion mutant Rev M10 negative control, pLJ ΔRev M10, in which the start codon was deleted. Stimulated lymphocyte populations were transduced by retroviral infection with supernatants derived from ψ-Crip amphotropic retroviral producer cells (vector titer on NIH 3T3 cells: $0.5–5.0 \times 10^6$ G-418-resistant colonies per ml). After transduction, cells were selected in G-418 for 5–7 days before challenge with HIV. Retroviral transduction frequencies for the lymphocyte population 3 days after transduction in anti-CD3/IL-2-stimulated PBL were estimated by limiting-dilution DNA PCR analysis. Immediately after transduction (days 1 and 2), 0.1–10% of cells contained the Rev M10 gene. Seven days after selection with G-418, the percentage of Rev-transduced cells in the populations, when tested, had increased to 10–60%. The relevant Rev M10 RNA was also detected in these cells by using RT PCR analysis, indicating that the vector successfully expressed its mRNA (data not shown).

To assess its biologic effects in these cells, cultures were challenged with a cloned laboratory isolate, HIV^{BRU}. A time course revealed a reduction in RT levels in Rev M10-transduced cells compared with ΔRev M10 negative controls (Fig. 1*A*). In addition, five independent experiments using lymphocytes from different HIV-seronegative donors revealed a consistent reduction in culture supernatant HIV RT levels in Rev M10 retrovirally transduced compared with the

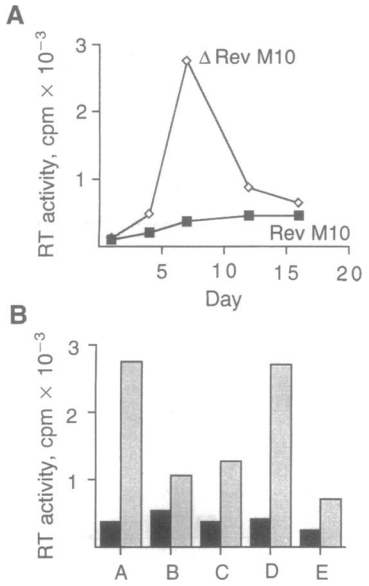

FIG. 1. Challenge of Rev M10/ΔRev M10 retrovirally transduced human PBL with HIV^{BRU}. (*A*) Representative time course of infection after challenge of Rev M10- and ΔRev M10-transduced PBL with HIV^{BRU}. (*B*) PBL from different donors were stimulated with phytohemagglutinin/IL-2 (groups A–C) or anti-CD3/IL-2 (groups D and E). After retroviral transduction with pLJ Rev M10 (black bars) or pLJ ΔRev M10 (gray bars), and G-418 selection for 1 week, cells were challenged with HIV. RT activity shown for each group is for 7–8 days post-HIV infection. Cells were challenged with HIV at a m.o.i. between 0.02 and 0.05. RT activity of PBL not exposed to G-418 ranged from 1.35- to 4.77-fold higher than pLJ ΔRev-transduced cells selected in its presence.

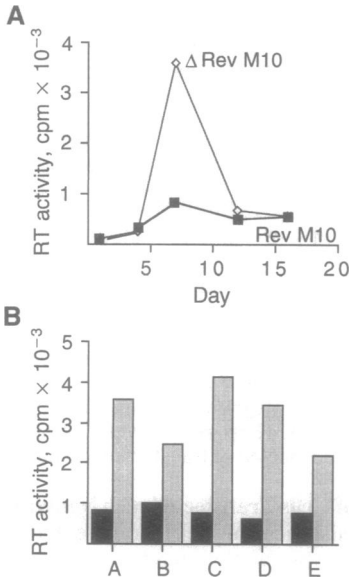

FIG. 2. Challenge of retrovirally transduced human PBL with a clinical isolate of HIV, HIV^{CLIN}. (*A*) Representative time course of infection after challenge of pLJ Rev M10- or pLJ ΔRev M10-transduced PBL with HIV^{CLIN}. (*B*) PBL from different donors were stimulated with phytohemagglutinin/IL-2 (groups A–C) or anti-CD3/IL-2 (groups D and E) as described. After retroviral transduction with pLJ Rev M10 or pLJ ΔRev M10 and G-418 selection for 1 week, cells were challenged with HIV^{CLIN}. Cells were then challenged with HIV at a m.o.i. between 0.02 and 0.05. RT activity shown for each group is for 7–8 days post-HIV infection, and RT levels of unselected cells ranged from 1.51- to 2.6-fold higher than pLJ ΔRev M10 cells selected in G-418. Black bars, Rev M10; gray bars, ΔRev M10.

ATG-deletion mutant control, ΔRev M10 (Fig. 1*B*). Although viral replication was suppressed in all cases, it was not completely abrogated, presumably because a proportion of cells did not contain the Rev M10 gene. Although RT levels were sometimes low (Fig. 1*B*), this effect was likely due to the extended period of culture and toxicity from retroviral supernatants. In every case where comparisons were made, however, RT levels correlated with p24 antigen levels (data not shown). These findings suggested that Rev M10 expression conferred relative protection from challenge with a cloned isolate of HIV. A 2- to 4-fold nonspecific inhibition of viral replication was also noted in cells grown in the presence of G-418 that had been transduced with the ΔRev M10 vector compared with unselected, nontransduced cells (Figs. 1 and 2 legends). This effect was likely due to the cytotoxic effect of G-418 on cell growth and viability or perhaps through its effect on Rev function (19).

To assess better the potential for Rev M10 to affect viral replication in infected patients, human PBL were challenged with freshly isolated HIV strains. Protection was also seen when cells were exposed to these viruses. A representative time course of HIV infection showed reduced RT levels in Rev M10 cultures compared with ΔRev M10 over the 14-day course of the experiment (Fig. 2*A*). In addition, supernatant RT levels at the peak of HIV infection were consistently reduced in multiple independent Rev M10-transduced cells with at least two independent freshly isolated HIV strains (Fig. 2*B*).

Particle-Mediated Gene Transfer into PBL: Transfection Frequencies and Integration Status. To explore the potential efficacy of a nonviral vector delivery system, particle-mediated gene transfer was used to introduce expression plasmids into human PBL. Plasmids encoding Rev M10 or the frameshift mutant lacking a start codon, ΔRev M10, under control of the Rous sarcoma virus promoter and the HIV tat-responsive element (TAR) (10) were used to transduce PBL, and the frequency of gene transfer was determined by limiting-dilution PCR. The percentage of cells initially transduced by this method was estimated to be at least 3% at 5 days after transduction and increased to ≥50% by 21 days of selection in G-418 (Table 1), and cells from HIV-seropositive donors were transduced with comparable frequencies (data not shown). The relevant Rev M10 RNA was also detected in these cells by using RT PCR analysis, suggesting that these vectors successfully synthesized their mRNA (data not shown). Cells could be maintained in the presence of G-418 for ≥2 mo, suggesting that the transfected gene could be stably incorporated in the genome of these cells. To confirm this hypothesis, Southern blot analysis was done. As early as 10 days after gene transfer, a signal corresponding to an integrated form of the recombinant Rev M10 gene was detected in human PBL, which was of comparable size to a stably transduced CEM line containing a single copy of this gene (Fig. 3). A second band, consistent with a linear free plasmid DNA form, could also be detected. The percentage of integrated DNA was estimated by densitometry to range from 11 to 46% of total in independent transfections 10 days after gene transfer and G-418 selection (Fig. 3).

Table 1. Transfection frequencies of particle-mediated gene transfer into PBL

Day	Transfection, %
5–8	3–20
14–16	>20
21	>50

Gene transfer frequencies for cells transfected by particle-mediated gene transfer were calculated by limiting-cell dilution PCR analysis using Rev-specific primers in normal PBL. PBL were transfected with Rev M10 expression plasmids as described.

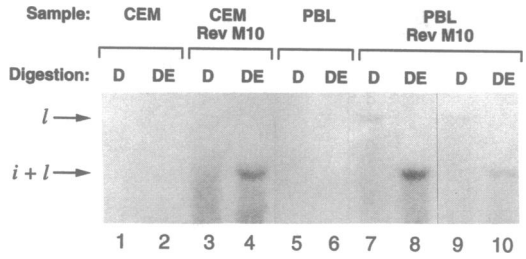

FIG. 3. Southern blot analysis of T leukemia and human PBL transfected with Rev M10 by particle-mediated gene transfer. Plasmids had been digested with *Xmn* I for linearization before introduction into cells. DNA samples were prepared and digested with *Dra* III (D) or *Dra* III and *Eco*RI (DE) to detect linearized free plasmid DNA (*l*) or integrated and linearized (*i* + *l*) forms of the plasmid of 3672 and 1142 bp, respectively. CEM (lanes 1–4) or PBL (lanes 5–10), untransfected or transfected as indicated, were probed.

Resistance of Cells Transfected with Nonviral Vectors to HIV Infection. Resistance of cells transfected with Rev M10 plasmid vectors by particle-mediated gene transfer was assessed after challenge with HIV^{BRU} or fresh clinical isolates. A time course following HIV^{BRU} challenge after transfection and G-418 selection revealed a reduction in RT levels in Rev M10-transfected cells compared with ΔRev M10 negative controls (Fig. 4*A*), similar to retrovirally transfected cells. Six independent experiments using lymphocytes from different HIV-seronegative donors also revealed a consistent reduction in culture supernatant HIV RT levels in Rev M10 compared with the ΔRev M10 control (Fig. 4*B*). As with retroviral vectors, replication was suppressed but was not completely abrogated because a proportion of cells did not contain the Rev M10 gene.

To assess the potential for Rev M10 to affect viral replication in fresh clinical isolates, PBL were transfected by the same method and challenged with recent isolates. Protection

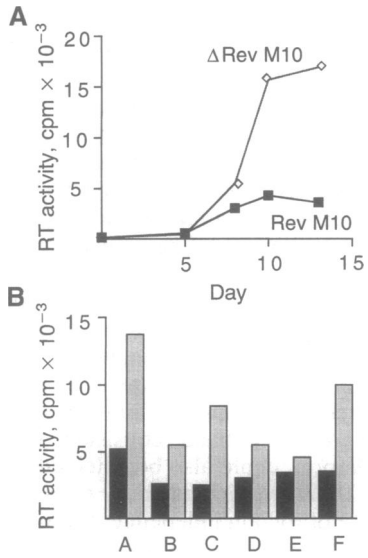

FIG. 4. Challenge of human PBL transfected by particle-mediated gene transfer with HIV^{BRU}. (*A*) A representative time course of HIV infection in PBL transfected with Rev M10 or ΔRev M10 expression vectors, pRSV/TAR Rev M10 or pRSV/TAR ΔRev M10, by particle-mediated gene transfer. (*B*) Experimental sets (A–F) of independently transfected PBL from separate donors were challenged with HIV^{BRU}. RT activity was determined 7–8 days post-HIV infection. Freshly isolated PBL were stimulated with anti-CD3/IL-2 as described. After particle-mediated transfection and selection in G-418 for up to 8 days, cells were challenged with HIV^{BRU} at a m.o.i. of between 0.02 and 0.05. Black bars, pRSV/TAR Rev M10; gray bars, pRSV/TAR ΔRev M10.

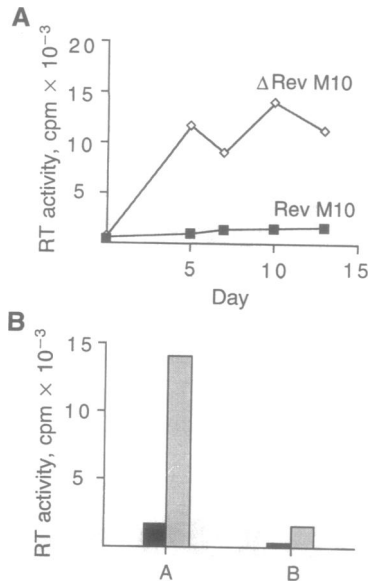

FIG. 5. Challenge of human PBL transfected by particle-mediated gene transfer with clinical isolates of HIV. (*A*) Representative time course of HIV infection of PBL transfected with Rev M10 or ΔRev M10 expression vectors, pRSV/TAR Rev M10 or pRSV/TAR ΔRev M10, by particle-mediated gene transfer. (*B*) Independently transfected PBL (*A* and *B*) were challenged with two different clinical isolates of HIV-1. RT activity was determined 7–8 days post-HIV infection. Freshly isolated PBL were stimulated with anti-CD3/IL-2 as described. After particle-mediated transfection and selection in G-418 for up to 8 days, cells were challenged with HIV^{CLIN} at m.o.i. values between 0.02 and 0.05. Black bars, Rev M10; gray bars, ΔRev M10.

was observed when cells were exposed to these viruses over the 14-day course of the experiments (Fig. 5*A*). In addition, supernatant RT levels at the peak of HIV infection were consistently reduced in independent Rev M10-transduced cells with two independent fresh isolated clinical strains (Fig. 5*B*).

DISCUSSION

Despite intensive efforts toward the development of treatments for AIDS, this disease has remained resistant to traditional pharmacologic intervention. For this reason, increased attention has turned to alternative approaches to interfere with the natural progression of HIV disease. Several gene products have now been described as effective in inhibiting HIV replication *in vitro*. These recombinant genes include RNA-based antiviral therapies, such as RNA decoys (20), ribozymes (21–24), and antisense approaches (25). In addition, several protein-based approaches have been successful in retarding the growth of HIV *in vitro*, including transdominant negative mutants such as the Rev M10 protein (6–10), Gag (26), envelope, and Tat mutants (27). More recently, successful antiviral effects have been achieved with intracellular antibody (single-chain variable-fragment expression) against viral gene products (28, 29). Despite the success of specific genes in inhibiting HIV replication in cell culture models, a major challenge remains the adaptation of this approach to clinical settings.

At least two major problems must be addressed for this approach to progress in human studies. These issues include (*i*) the effective delivery of recombinant genes into primary human T cells and other cell types and (*ii*) the ability to introduce these genes safely and without activation of endogenous HIV within the seropositive patient cell population. In this study, we have developed approaches that begin

to address these problems. In the first case, we have developed a nonviral DNA delivery system as an alternative to retroviral gene transfer. A major concern regarding the use of viral vector delivery systems is the potential for small quantities of replication-competent virus to remain undetected in large production lots intended for clinical use. Because it is not possible to test the entire lot, quantities below the detection limits of the assay could be introduced into the patient. Given the underlying immunodeficiency of these patients, the potential to establish replication-competent virus in the host would be increased. Although proper testing of retroviral vector batches makes this possibility less likely, it remains a risk in this procedure. The use of a nonviral vector that is unable to replicate provides an alternative to this approach that might improve its safety. A variety of approaches have been attempted to introduce genes into T cells but most are limited by low transfection efficiencies, transient expression, and the general resistance of T cells to the uptake of recombinant DNA.

In this study, a particle-mediated gene transfer method was used, and protocols were modified to achieve optimal transfection frequencies within T cells. With this technique, between 2 and 10% of cells were routinely transfected. In general, the rates of gene transfer were up to ≈10-fold higher than those achieved with retroviral vectors. Although the presence of transfected DNA was detected soon after gene transfer, cells could be maintained and selected for long periods of time (>2 mo) in cell culture, indicating the stability of expression. This stability was confirmed by Southern blotting of cell lines, which revealed a signal from integrated DNA, detectable as early as 10 days after transfection and selection *in vitro*. This nonviral delivery method, as well as retroviral vectors, showed greater efficacy when T cells were stimulated *in vitro* before gene transfer.

This gene delivery approach can potentially be applied to other relevant target cells, including hematopoietic stem cells and dendritic cells. Whether appropriate expression of HIV protective genes in these target cell populations can be achieved is currently unknown. It is possible, however, to generate expression vectors for these HIV protective genes whose inhibition of viral spread can be optimized by appropriate regulation of gene expression (10). These techniques will allow evaluation of the efficacy of a variety of antiviral genes for treatment of HIV infection in patients (30). At the same time, the ability to introduce eukaryotic expression vectors into human primary PBL by these methods will enable further analysis of the pathways of T-cell activation responsible for activation of specific transcription factors within these cells. Taken together, these results should provide further insight into basic questions regarding T-cell gene expression and have practical implications for gene transfer relevant to the treatment of HIV infection.

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