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Expression of a protective gene prolongs survival of T cells in human immunodeficiency virus-infected patients

(gene therapy/Rev/transdominant/biolistics)

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ABSTRACT **The resistance of acquired immunodeficiency syndrome (AIDS) to traditional drug therapy has prompted a search for alternative treatments for this disease. One potential approach is to provide genetic resistance to viral replication to prolong latency. This strategy requires the definition of effective antiviral genes that extend the survival of T cells in human immunodeficiency virus (HIV)-infected individuals. We report the results of a human study designed to determine whether a genetic intervention can prolong the survival of T cells in HIV-infected individuals. Gene transfer was performed in enriched CD4$^+$ cells with plasmid expression vectors encoding an inhibitory Rev protein, Rev M10, or a deletion mutant control, ΔRev M10, delivered by gold microparticles. Autologous cells separately transfected with each of the vectors were returned to each patient, and toxicity, gene expression, and survival of genetically modified cells were assessed. Cells that expressed Rev M10 were more resistant to HIV infection than those with ΔRev M10 *in vitro*. In HIV-infected subjects, Rev M10-transduced cells showed preferential survival compared to ΔRev M10 controls. Rev M10 can therefore act as a specific intracellular inhibitor that can prolong T-cell survival in HIV-1-infected individuals and potentially serve as a molecular genetic intervention which can contribute to the treatment of AIDS.**

Replication of the human immunodeficiency virus (HIV) is dependent on interactions between essential viral regulatory proteins and cellular factors (1). Because infected cells contain acquired genetic information from HIV, viral genes represent logical genetic targets to inhibit HIV replication, and the use of intracellular inhibition or immunization has been proposed as a potential means to interfere with viral replication (2–4). Inhibition of HIV replication has now been established by using different antiviral genes, including those directed at RNA or protein targets (5–20).

Among HIV gene products, the Rev protein is translated from a highly spliced viral mRNA synthesized early in the course of virus infection. Rev affects viral latency in chronically infected cells and is essential for productive viral replication (20–23). Mutation of a highly conserved leucine-rich domain that interacts with cellular factors gives rise to a defective protein that acts as an inhibitor of HIV replication and does not affect normal T-cell function (6–8, 24). Specifically, amino acid substitutions of aspartic acid for leucine and leucine for glutamic acid at positions 78 and 79, respectively, generate a protein that binds to the Rev-responsive element competitively with wild-type Rev (22). Rev M10 thus represents one of a number of antiviral genes effective in cell culture, but the ability of such genes to function in HIV-infected humans was unknown. Such efficacy and associated safety issues required additional human studies to understand their potential utility *in vivo*. To address these questions, a study to define the potential of antiviral genes to function *in vivo* was performed in HIV-infected individuals.

MATERIALS AND METHODS

Patient Selection. Three patients who satisfied the entry criteria of the study (25) were treated in the Clinical Research Center at the University of Michigan Medical Center. In each case, these patients had CD4 counts of 400–500 cells per mm^3 upon enrollment. Each patient tolerated the treatments well, with no complications or adverse events.

Gene Transfer. Patient peripheral blood leukocytes (PBLs) (6×10^9 cells) were collected by apheresis at the University of Michigan Medical Center General Clinical Research Center. After separation on Ficoll/Hypaque gradients, 4×10^9 cells were depleted of CD8$^+$ cells by binding to 175-cm^2 CD8-antibody-coated plates (Applied Immune Sciences, San Jose, CA). CD4-enriched PBLs suspended in X-Vivo 15 medium (BioWhittaker) were stimulated for 48 hr on plates coated with OKT-3 (Ortho) at 10 μg/ml in the presence of 5 μM delavirdine (U90152S) (26) and 4 nM CD4-PE40 (27) (Upjohn). After stimulation, equal numbers of cells were transduced with the linearized Rev M10 or ΔRev M10 Rous sarcoma virus (RSV) expression plasmids by particle-mediated gene transfer as described (9). Patient 1 cells (10^9 cells per vector) underwent the gene transfer procedure twice, once a day on two consecutive days. Cells from patients 2 and 3 were transduced once, but patient 2 received two additional cell transfers of 10^{10} cells per vector (≈10% transfection efficiency) when signals were found to be barely detectable after transfer of 10^9 cells (see Fig. 5). Transduced populations were grown in X-Vivo 15 medium containing the antiviral agents and interleukin 2 (Chiron; 300 international units/ml) in Lifecell Tissue culture flasks (Baxter). Cultures were expanded up to 10 days. One day prior to infusion, culture supernatants were assayed for p24 antigen (Coulter) and for the presence of bacterial/fungal/mycoplasma contaminants. In no case was p24 antigen detected above background nor were contaminants detected. Immediately prior to reinfusion cells were washed four times with Dulbecco's PBS to eliminate medium and antiviral agents. After counting, equal numbers of Rev M10- and ΔRev M10-transduced PBL populations were mixed together and resuspended in 100–150 ml of saline containing 450,000 units of interleukin 2 and 1.25% human albumin. The cells were reinfused back into the patient over a 30-min period.

Analysis of Plasmid in Transfected Cells. Southern blot analysis was performed as described for genetically modified cells (9). Restriction enzyme digestions are described in Fig. 2*B*. Plasmid copy number per cell was estimated by comparison of counts compared to a single copy gene, the p21 cyclin-

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Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; PBL, peripheral blood leukocyte.

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dependent kinase inhibitor by PhosphorImager analysis (Molecular Dynamics) and normalized for transfection efficiency, ≥10% for each patient, by limiting-dilution PCR (polymerase chain reaction). Exposure of Southern blot for p21 was 3 days, compared to 12 hr for RSV/TAR Rev M10 or ΔRev M10.

PCR Analysis. PCR analysis of genetically modified cells was performed by using a common radiolabeled primer pair that detects both vectors (see Fig. 4*C*) under standard conditions (91°C for 1 min and at 72°C for 20 sec for 42 cycles). Samples were analyzed after electrophoresis on a nondenaturing 7% polyacrylamide gel and signals were quantitated with a Molecular Dynamics PhosphorImager.

Reverse Transcriptase-Coupled PCR (RT–PCR) Analysis. Total RNA from PBLs stably transduced with either vector was isolated with Trizol reagent (GIBCO/BRL) according to the manufacturer's directions. RT–PCR was performed according to the manufacturer's instruction (Perkin–Elmer). Four of the 5 bp at the 3′ end of the RT primer were not complementary to the wild-type HIV Rev sequence; hence, wild-type HIV Rev mRNA was not reverse-transcribed by this primer (see Fig. 4*C*). Fourteen base pairs at the 3′ end of this primer are complementary to *rev* M10, and an additional 17 bp were added as a 5′ overhang (5′-aataagtcggcacgcgcGTAAGTCTCAGATC-3′). Since the additional 17 bp are not present in the genomic or vector DNA, only the cDNA is amplified in the subsequent PCR using the RT primer as the antisense primer at a higher annealing temperature. RT–PCR was performed individually in a single vial, avoiding the transfer step (28). The RT reaction was performed in a total volume of 10 μl that was diluted 1:5 with *Taq* reaction mixture. The RT reaction mixture contained 5 mM Mg^{2+}, all four dNTPs (each at 1.25 μM), and the RT primer at 1.3 μM. For RT reaction, the vials were incubated at 37°C for 10 min, 42°C for 15 min, and 95°C for 5 min. The conditions for amplification were denaturation at 91°C for 1 min, and annealing/extension at 72°C for 1 min for a total of 30 cycles. The amplified material was visualized by ethidium bromide staining on a 1% agarose gel (see Fig. 4*A*). For Fig. 4*B*, PCR products were identified by autoradiography after hybridization to an end-labeled probe (5′-AATGGGAGGTGGGTTGCTTTGATAGAGAAACTT-3′) using Quick Hyb reagent (Stratagene) after transfer to a GeneScreen membrane (NEN) by passive capillary transfer.

RESULTS

Plasmids encoding Rev M10 or ΔRev M10 under control of the RSV promoter and the HIV tat responsive element (TAR) were introduced separately into equal numbers of $CD4^+$-enriched cells from each patient (Figs. 1 and 2*A*; see also ref. 20) by using gold-particle-mediated gene transfer after linearization by restriction enzyme digestion (9). The frequency of gene transfer, determined by limiting dilution PCR, was consistently ≥10% (9). Southern blot analysis revealed signals corresponding to 10^2–10^4 copies of the unintegrated forms of Rev M10 or ΔRev M10 per transfected cell that received plasmid DNA (Fig. 2 *B* and *C*). Although a large percentage of transduced cells showed a high copy number of plasmid DNA, a large percentage of DNA was lost during cell culture over a 10-day period. At the time of cell infusion, a 10^2- to 10^3-fold reduction of plasmid DNA per cell was found, with a substantial increase in the percentage (≥80%) of integrated DNA (Fig. 2*C*). Such cells can be stably maintained *in vitro* under drug selection (9). No differences were observed in growth or gene transfer efficiency of these cell populations during *ex vivo* expansion (ref. 24; see also Fig. 5). Similarly, the percent integration was relatively constant regardless of transfection efficiency (data not shown), and no adverse effects have been detected in cell viability and function *in vitro* (24).

To confirm the antiviral effects of Rev M10 in patient cells, Rev M10 and ΔRev M10 cells were maintained separately *in vitro*, enriched by selection in G418, and challenged with HIV^{BRU} or HIV^{IIIB}. A time course revealed a reduction in p24 antigen levels in infected Rev M10-transduced cells compared to ΔRev M10 negative controls in all patients (Fig. 3). RT analysis confirmed this protection; a 4- and 5.5-fold reduction in RT levels at day 7 was seen for patients 1 and 3, respectively, although as observed in previous studies, protection could be overcome at higher multiplicities of infection (9). The sensitivity of these cell lines to G418 selection was similar, suggesting that differences in viral replication were not related to levels of the neomycin phosphotransferase protein but to expression of Rev M10. Expression of Rev M10 in patient cells, therefore, provided antiviral effects *in vitro*. The expression of Rev M10 or ΔRev M10 mRNA was confirmed *in vivo* by analysis of PBLs. PBLs were isolated from patients who had been reinfused with Rev M10- and ΔRev M10-transduced $CD4^+$ cells, and RT–PCR analysis was performed prior to infusion or 1 hr after gene transfer. Primers common to Rev

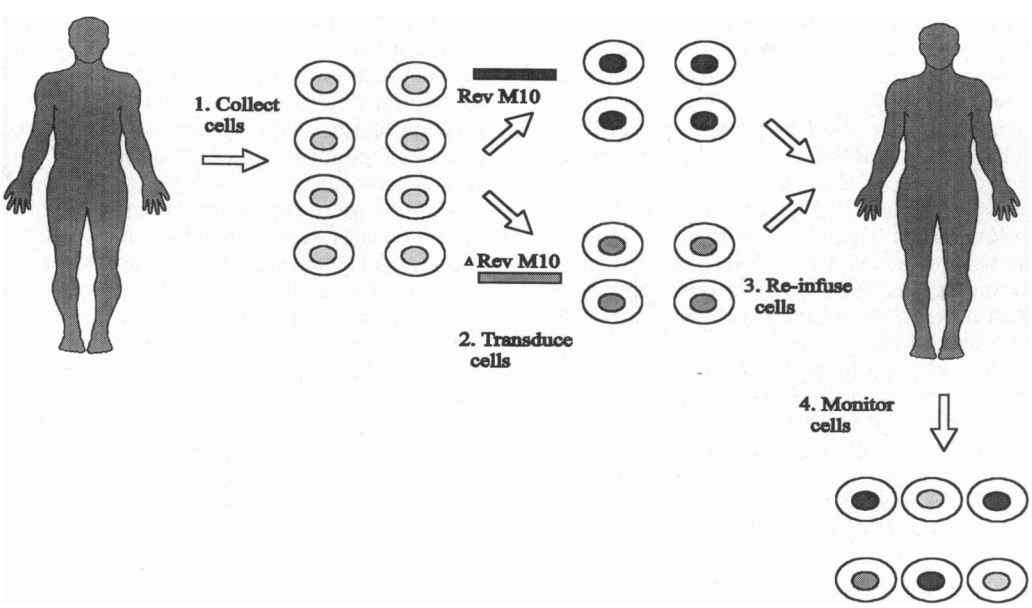

FIG. 1. Schematic representation of the human clinical protocol.

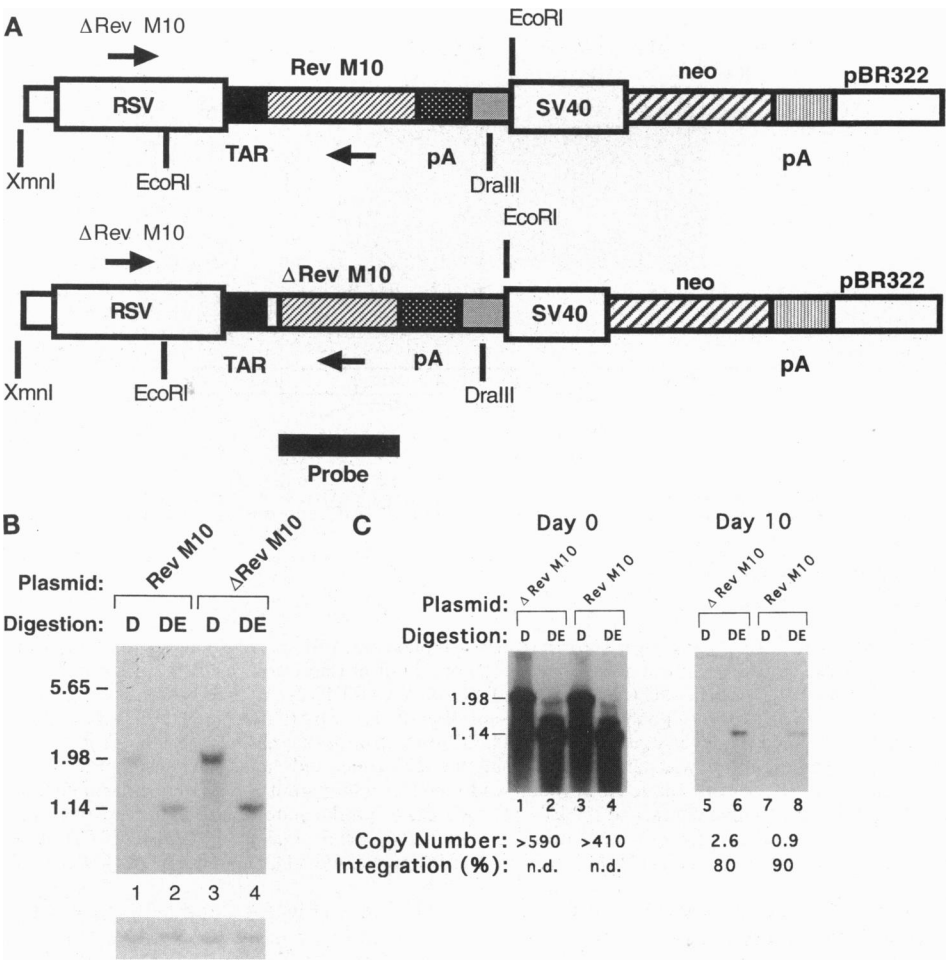

FIG. 2. Structure of vectors and Southern blot analysis of DNA in genetically modified patient cells. (*A*) Structure of pRSV/TAR Rev M10, which expresses the Rev M10 open reading frame, and a nearly identical plasmid pRSV/TAR ΔRev M10, with a small internal 13-bp deletion used as a negative control in the gene transfer protocol (9, 20, 25). Arrows denote sites of PCR primers, and relevant restriction enzymes are shown. (*B*) Analysis of recombinant DNA in patient cells prior to reinfusion by Southern blots. $CD4^+$ cells derived from patient 1 were genetically modified with the indicated vectors after stimulation with CD3 monoclonal antibody for 2 days (9). Immediately prior to mixing and reinfusion, samples transfected with the Rev M10 (lanes 1 and 2) or Rev ΔM10 (lanes 3 and 4) vector were removed for Southern blot analysis (34). Plasmids had been digested with *Xmn* I for linearization prior to introduction into cells. DNA samples were prepared and digested with *Dra* III (*D*) to detect concatamers (5653 bp) or linearized free plasmid DNA (1985 bp) or digested with *Dra* III and *Eco*RI (DE) to detect all forms of the plasmid (1142 bp) in $CD4^+$-enriched PBLs. A single copy gene, p21 cyclin-dependent kinase inhibitor, is shown at the bottom. Similar results were observed for patients 2 and 3. (*C*) Analysis of DNA copy number and integration in patient 2 whose cells were transfected with pRSV/TAR ΔRev M10 (lanes 1, 2, 5, and 6) or pRSV/TAR Rev M10 (lanes 3, 4, 7, and 8). DNA was analyzed at day 0 (lanes 1–4) or day 10 (lanes 5–8) as described above (Fig. 1*B*). Copies per transduced cell and percent integration were quantitated by densitometry and compared to the intensity of a single copy gene, p21, detected by reprobing the same blot after washing. n.d., Not determined.

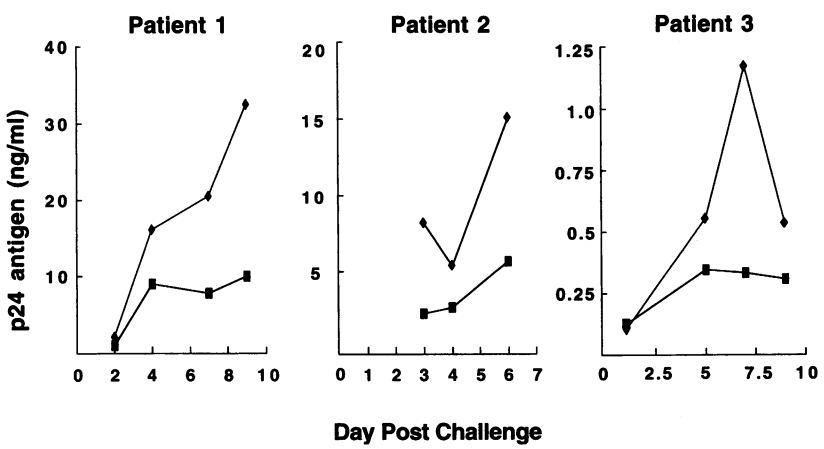

FIG. 3. Antiviral effect of Rev M10 in patient cells *in vitro*. $CD4^+$ cells from each patient were transfected with pRSV/TAR Rev M10 or pRSV/TAR ΔRev M10, respectively. After particle-mediated transduction and selection in G418 for 8 days, cells were challenged with HIV^{IIIB} (patients 1 and 3) at a multiplicity of infection between 0.005 and 0.01 or HIV^{BRU} (patient 2) at a multiplicity of infection of 0.05. After HIV infection for 2 hr followed by washing once, p24 antigen levels (Coulter) were assayed in duplicate from culture supernatants at the indicated times. Cells were purified by Ficoll/Hypaque separation and CD8-depleted with antibody-coated plates (provided by Applied Immune Science, Santa Clara, CA), prior to transduction.

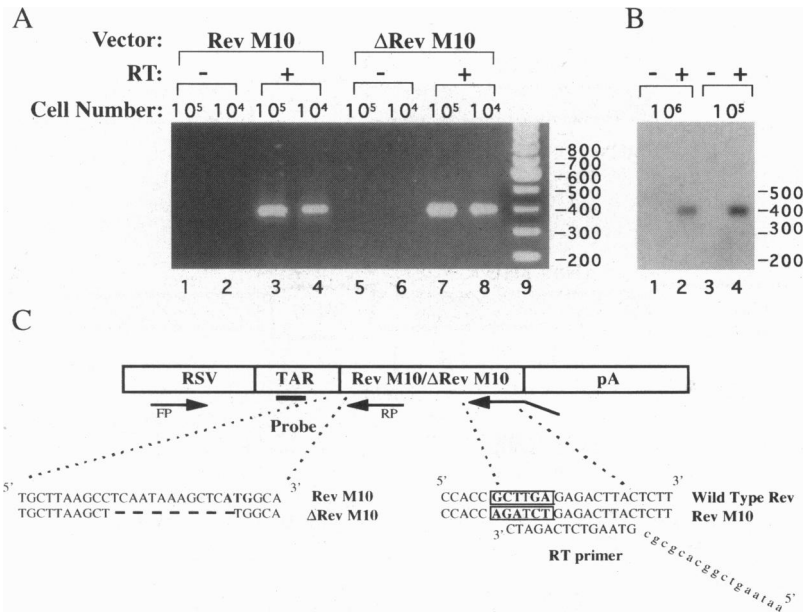

FIG. 4. Detection of Rev M10 and ΔRev M10 vector RNA in patient lymphocytes. PBLs were isolated from patients and genetically modified with the indicated vectors and analyzed prior to infusion by RT–PCR (*A*) or 1 hr after reinfusion (*B*) by RT–PCR and probing with a radiolabeled Rev M10 oligonucleotide (5′-AATGGGAGGTGGGTTGCTTTGATAGAGAAACTT-3′). Total RNA was incubated in the presence (+) or absence (−) of RT and analyzed by PCR (34). (*C*) Schematic representation of the primers used for DNA PCR and RT–PCR. DNA PCR was performed by using the forward (FP) and the reverse primers (RP). The 13-bp sequence that has been deleted from Rev M10 to make ΔRev M10 is shown as a dashed line. Reverse transcription was performed by using the RT primer and the cDNA was amplified with the combination of FP and RT primers. The difference in the nucleotide sequence between wild-type Rev and mutant Rev M10 is shown in boldface type (boxed region). The 14 bp at the 3′ end of the RTP are complementary to the Rev M10 sequence and the added 17-bp noncomplementary sequence at the 5′ end is shown in lowercase type. The sequences of the oligonucleotides are as follows (all 5′ → 3′): FP, CGCCATTTGACCATTCACCACATTG-GTGTGCA; RP, TTGAGGAGGTCTTCGTCGCTGTCTCCGCTTCTT; Probe, CCAGATCTGAGCCTGGGAGCTCTCTGGCTA.

M10 and ΔRev M10 expression were used, giving rise to fragments in the presence of RT of the predicted size that hybridized to an internal *rev* probe, thus documenting the expression of the recombinant gene after cell transfer *in vivo* (Fig. 4*B*).

The persistence of genetically modified cells was determined by performing limiting dilution PCR in PBL samples obtained at different times after gene transfer. Since the Rev M10 and ΔRev M10 vectors were identical except for the small 13-bp deletion eliminating the ATG, common primers were again used to generate products of known size at each cell dilution. The combination of competitive and limiting dilution PCR analysis thus allowed independent verification of the frequency of gene-modified cells *in vivo*. No recombinant genes were detected in PBLs from any patients who received 10^9 total cells except for very weak signals at the limit of sensitivity (10^{-6}) 1 hr after infusion. Two of these patients, patients 2 and 3, were subsequently infused with 2×10^{10} cells, of which ≥10% were genetically modified and contained equal numbers of Rev M10- or ΔRev M10-transfected cells (Fig. 5). The percentage of genetically modified cells did not change substantially during *in vitro* expansion so that nearly equal numbers of Rev M10 and ΔRev M10 cells were infused (Fig. 5). Equivalent signals for Rev M10 and ΔRev M10 were seen 1 hr after gene transfer at frequencies of $\geq 10^{-5}$ in both patients. After 1 week, the relative ratio of Rev M10- to ΔRev M10-transduced cells increased 10-fold in both patients (Fig. 5*A Left* and *Right*), although the absolute number of genetically modified cells decreased in patient 2. Because the survival of Rev M10-transduced cells in patient 2 was less long-lived, a second gene transfer protocol was performed in this patient. A similar protective effect was observed in this patient after this treatment (Fig. 5*A Center*). Patient 3, in particular, maintained Rev M10- but not ΔRev M10-transduced cells for a longer period of time at frequencies of 10^{-4} after 2 weeks, at 10^{-5} at 4 weeks, and 10^{-6} at 8 weeks (Fig. 5*B*). In this patient, Rev M10 cells showed an ≈15-day half-life, compared to 3.5 days for ΔRev M10 cells (Fig. 5*A*), and a 10- to 100-fold difference in these populations was seen from days 7 through 28. The half-life of Rev M10 cells was 4 days compared to ≤1 day in ΔRev M10 cells in patient 2 (data not shown).

These data suggest that genetically modified cells can be detected, and the cells expressing Rev M10 demonstrated a 4- to 5-fold selective survival advantage. Patients were also monitored for a variety of serum biochemical, hematologic, immune, and virologic parameters. In all patients, no new abnormalities resulted from the treatment, and no increase in detectable serum p24 antigen or HIV RNA by the bDNA method (29) was observed after transfer of 10^{10} cells (data not shown).

DISCUSSION

In this study, we have begun to assess the feasibility of gene transfer as a potential intervention for AIDS. Although a variety of recombinant genes have been used successfully to prevent HIV replication *in vitro*, a major challenge remains the adaptation of gene transfer *in vivo*, the definition of effective antiviral genes in HIV-infected patients, and a determination of the potential toxicity and efficacy of this approach in clinical settings. We find that Rev M10 prolonged the survival of genetically modified cells, likely protecting them through its ability to inhibit productive viral replication in these cells (Fig. 3).

These cells are detectable transiently in some patients, and several factors may contribute to this finding. (*i*) The *ex vivo* culture of T cells may affect *in vivo* trafficking, and activated T cells may migrate from the circulation to peripheral lymphoid tissue. (*ii*) The percentage of cells transferred in these marking studies after the infusion (≈10^{-5}) is at the limit of sensitivity of the PCR (10^{-5}–10^{-6}) and can fall easily below the limits of detection. (*iii*) The recent finding of rapid T-cell turnover in HIV-infected patients suggests that nonspecific cell loss is to be expected in AIDS patients (30–32). In the future, it is likely that cell engraftment and expansion can be

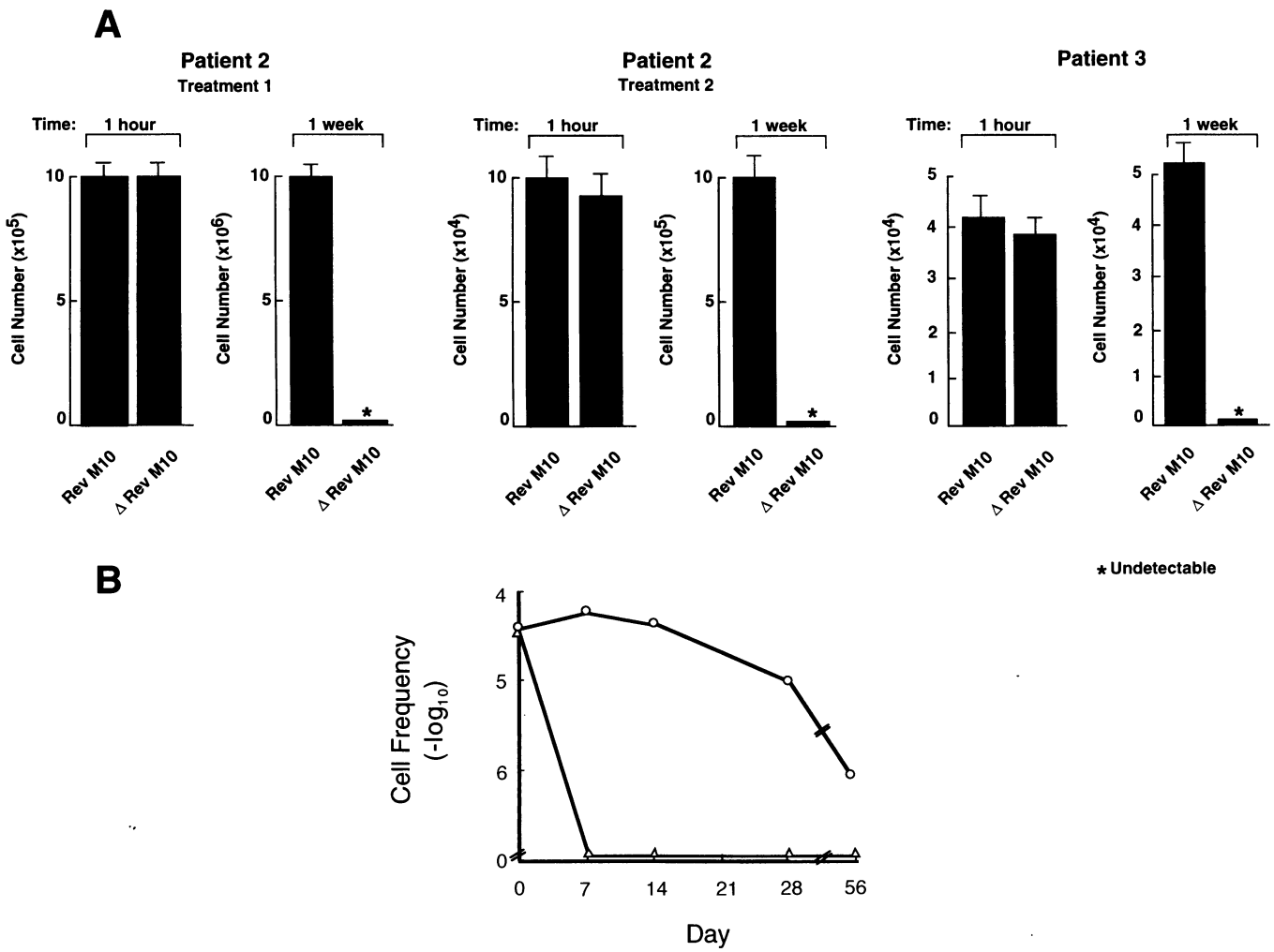

FIG. 5. Prolonged survival and kinetics of Rev M10-transduced cells in patients. (*A*) PBLs were isolated from patient 2, treatment 1 (*Left*); patient 2, treatment 2 (*Center*); or patient 3, treatment 2 (*Right*) 1 hr or 1 week after cell infusion and analyzed by limiting dilution PCR. (*B*) A time course of cell survival in patient 3 by PCR analysis is shown. Survival of each cell type is plotted on a logarithmic scale (below). In each patient, a total of 10^9 CD4-enriched cells were transfected (data not shown), and similar numbers of Rev M10 and ΔRev M10 populations were expanded prior to reinfusion [patient 2 (treatment 1), ΔRev M10 (1×10^9 cells), Rev M10 (1×10^9 cells); patient 2 (treatment 2), ΔRev M10 (1.2×10^{10} cells) and Rev M10 (1.3×10^{10} cells); patient 3, ΔRev M10 (1.15×10^{10} cells) and Rev M10 (1.13×10^{10} cells)]. Transfection efficiencies were ≈10% in all cases and relative ratios were unchanged after *in vitro* expansion. Patient 1 was discontinued from the protocol for personal reasons unrelated to the treatment and before any additional samples of the higher dose were collected.

significantly improved in efforts to develop clinical efficacy. This goal may be achieved through the use of a larger number of genetically modified cells or through adjunct treatments, such as the administration of growth-promoting cytokines (e.g., interleukin 2) as shown in HIV-infected individuals (33). In addition, the use of antiviral drugs may also prolong T-cell survival by reducing viral burden (30, 31) and help to promote their growth and persistence *in vivo*. Combinations of antiretroviral agents and immunotherapeutic strategies may be best suited to promote establishment of genetically protected cells *in vivo*.

Among recombinant genes with potential antiviral activity *in vitro* are antiviral RNAs such as decoys (10), ribozymes (11–14), antisense RNAs (15), or antiviral proteins such as dominant-negative Rev (6–9), GAG, envelope (16), Tat mutants (17), or intracellular antibodies (18, 19). A major challenge to the development of molecular genetic interventions for AIDS is the adaptation of genetic strategies to clinical settings. The study design in the present report provides a clinically adaptable method to achieve gene transfer and to analyze the potential efficacy of antiviral genes (Fig. 1). This protocol allows an assessment of safety and toxicity at the same time that genetic analysis can be used to define the effects of Rev M10 and other potentially protective recombinant genes.

Using HIV-specific antiretroviral agents, autologous cells could be used safely in this study, potentially allowing treatment in a large number of patients. Previous studies did not use such HIV-specific agents, and for safety reasons, were performed by using gene transfer in identical twins, from uninfected to infected siblings. The lack of toxicity and absence of p24 antigenemia after T-cell gene transfer suggests that marking studies can be used safely to assess the effects of different antiviral genes on cell survival in autologous cells. Additional studies will help to define clinical efficacy and to optimize methods of T-cell activation for gene transfer. This approach will also help to identify effective combinations of antiviral genes to use in other hematopoietic cells for immune reconstitution with protected cells. Quantitative comparison of expression and antiviral effect in genetically altered cells of HIV-infected individuals will facilitate efforts to understand the genetic determinants of viral infection in humans, the kinetics of viral and cell replication, and the potential of molecular genetic interventions in AIDS patients.

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