

# Inhibition of Clinical Human Immunodeficiency Virus (HIV) Type 1 Isolates in Primary CD4<sup>+</sup> T Lymphocytes by Retroviral Vectors Expressing Anti-HIV Genes

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Received 23 December 1994/Accepted 4 April 1995

**Gene therapy may be of benefit in human immunodeficiency virus type 1 (HIV-1)-infected individuals by virtue of its ability to inhibit virus replication and prevent viral gene expression. It is not known whether anti-HIV-1 gene therapy strategies based on antisense or transdominant HIV-1 mutant proteins can inhibit the replication and expression of clinical HIV-1 isolates in primary CD4<sup>+</sup> T lymphocytes. We therefore transduced CD4<sup>+</sup> T lymphocytes from uninfected individuals with retroviral vectors expressing either HIV-1-specific antisense-TAR or antisense-Tat/Rev RNA, transdominant HIV-1 Rev protein, and a combination of antisense-TAR and transdominant Rev. The engineered CD4<sup>+</sup> T lymphocytes were then infected with four different clinical HIV-1 isolates. We found that replication of all HIV-1 isolates was inhibited by all the anti-HIV vectors tested. Greater inhibition of HIV-1 was observed with transdominant Rev than with antisense RNA. We hereby demonstrated effective protection by antisense RNA or transdominant mutant proteins against HIV-1 infection in primary CD4<sup>+</sup> T lymphocytes using clinical HIV-1 isolates, and this represents an essential step toward clinical anti-HIV-1 gene therapy.**

The ultimate goal in AIDS research is to develop effective strategies that can inhibit human immunodeficiency virus type 1 (HIV-1) gene expression or function and consequently limit HIV-1 replication and AIDS pathogenesis. Current anti-HIV-1 therapies, such as reverse transcriptase inhibitors, can effectively inhibit virus replication but have shown little long-term clinical benefit (10). Since HIV-1 integrates into the genome of its target cell, AIDS can be considered an acquired genetic disease and as such is potentially amenable to gene therapy strategies. Many different anti-HIV gene therapy approaches have been developed to inhibit the virus at the intracellular or extracellular level (reviewed in references 19, 39, 53, and 56). HIV-1 may be inhibited by targeting HIV-1 proteins or *cis*-acting regulatory sequences. These anti-HIV-1 gene therapy strategies can be either DNA based (antisense oligonucleotides), RNA based (antisense-RNA, RNA decoys, and ribozymes), or protein based (transdominant HIV-1 proteins, gene vaccines, "suicide" proteins, and cellular anti-HIV proteins).

A major limitation of most anti-HIV gene therapy studies is that the efficacy of these strategies has been assessed almost exclusively with HIV-1 laboratory strains and T-cell lines. In addition, it is difficult to evaluate which anti-HIV strategy or vector is most effective, since side-by-side comparisons have not been performed. To overcome these present limitations, we have compared in this study the efficacies of different anti-HIV-1 vectors under conditions relevant for clinical gene therapy. These conditions included testing with primary CD4<sup>+</sup> T

lymphocytes instead of T-cell lines, along with challenge by clinical HIV-1 isolates. The clinical HIV-1 isolates used in the present study were directly isolated from HIV-1-infected individuals and included strains that are resistant to the most commonly used anti-HIV drug, 3'-azido-3'-deoxythymidine (AZT).

Several therapeutic approaches, such as use of soluble CD4 and HIV-1 vaccines, that worked well against cell-line-adapted HIV-1 laboratory strains have previously been proven ineffective against clinical HIV-1 isolates in primary cells by virtue of the polymorphic nature of the HIV-1 Env protein (16, 43). Hence, it is important to develop gene therapy strategies aimed at inhibiting the function of conserved HIV-1 proteins, such as Tat and Rev. We therefore developed four different anti-HIV-1 retroviral vectors aimed at inhibiting Tat and Rev function to test the efficacy of anti-HIV gene therapy in primary T cells. The HIV-1 Tat and Rev genes encode essential regulatory proteins that mediate transactivation of HIV-1 gene expression by binding onto the HIV-1 mRNA TAR (Tat transactivation-responsive element) and RRE (Rev-responsive element) sequences, respectively. The Tat-TAR interaction leads to a potent transactivation of viral gene expression by inducing transcriptional initiation and/or elongation (4, 11, 18, 24, 41, 52). The Rev-RRE interaction also strongly transactivates HIV-1 by facilitating the extranuclear transport of unspliced and singly spliced mRNAs that encode HIV-1 structural proteins (17, 23, 33, 52). Inhibition of Tat and/or Rev function may therefore preclude HIV-1 replication and gene expression. It has been speculated that certain HIV-1 gene products may be associated with virus-independent pathology. For example, in addition to acting on HIV-1 itself, the Tat protein may also participate in the pathogenesis of other AIDS-associated disorders such as Kaposi's sarcoma (15), B-cell lymphomas (25), and nervous-system disorders (46). Further, a number of disease-related manifestations are associated with the Rev-de-

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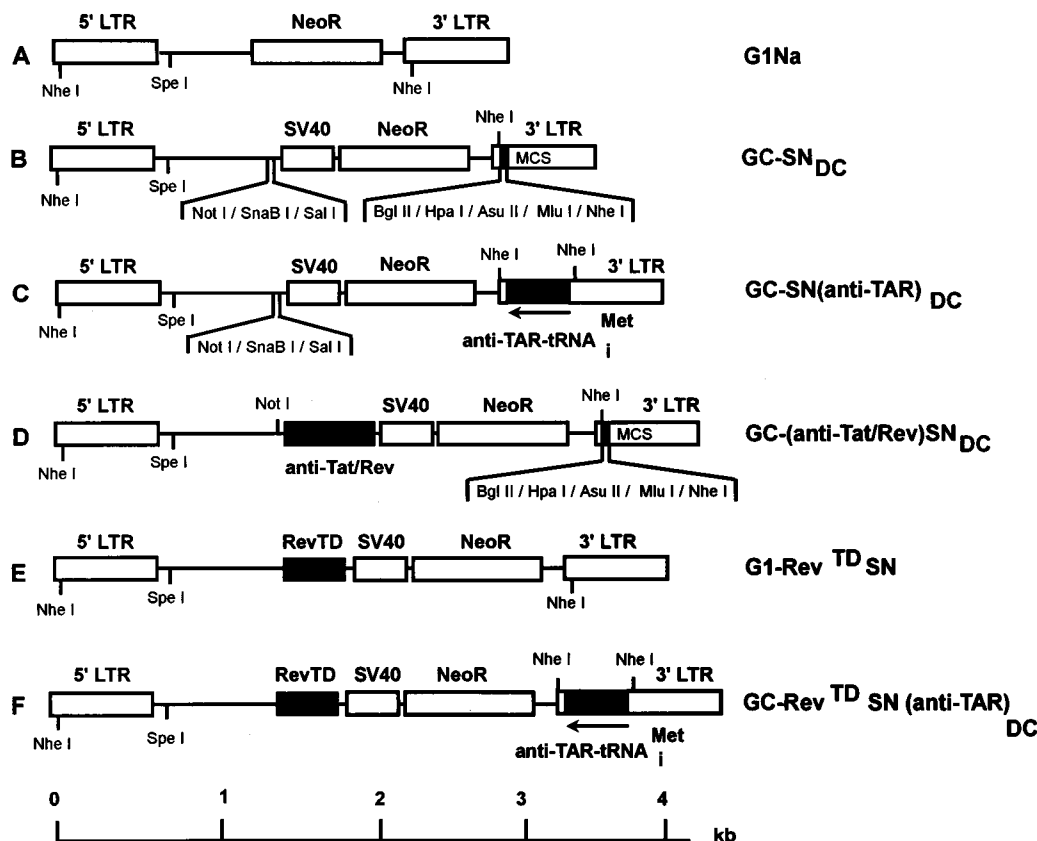


FIG. 1. Maps of the various retroviral vectors, G1Na (A), GC-SN<sub>DC</sub> (B), GC-SN(anti-TAR)<sub>DC</sub> (C), GC-(anti-Tat/Rev)SN<sub>DC</sub> (D), G1Rev<sup>TD</sup>SN (E), and GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub> (F). The MCS within the 3' LTR is shown. The neomycin resistance gene (NeoR) is driven by the SV40 early promoter in all vectors except G1Na, in which it is driven by the MoMuLV 5' LTR. The antisense-Tat/Rev (anti-Tat/Rev) and Rev<sup>TD</sup> genes are also driven by the MoMuLV 5' LTR. The antisense-TAR (anti-TAR) gene was cloned as a tRNA<sup>Met</sup>-anti-TAR fusion gene into the 3' LTR.

pendent expression of the HIV-1 envelope gene (gp120). These virus-independent, gp120-related pathologies included uncontrolled lymphocyte proliferation (38), activation-induced apoptosis (3, 31), and potential neuronal damage (21). Gene therapy may be a clinically applicable method for the specific down-regulation of HIV-1 gene expression.

The retroviral vectors that were used in this study were designed to express either antisense-TAR RNA, antisense-Tat/Rev RNA, or transdominant Rev (Rev<sup>TD</sup>) proteins. Rev<sup>TD</sup> contains a point mutation (Leu-78 to Asn-78) in the leucine-rich C-terminal domain, important for activation of Rev function. Consequently, it lacks intrinsic wild-type activity and inhibits the function of its cognate wild-type protein in *trans* (44a). Herein we report that HIV-1 isolates obtained from infected individuals could be inhibited in primary CD4<sup>+</sup> T lymphocytes by using retroviral vectors expressing antisense-TAR, antisense-Tat/Rev, and Rev<sup>TD</sup> proteins. We further show that HIV-1 inhibition by the Rev<sup>TD</sup> vectors was more potent than it was by the antisense vectors.

## MATERIALS AND METHODS

**Vector construction.** The G1Na Moloney murine leukemia virus (MoMuLV)-derived retroviral vector (Fig. 1A) expresses the neomycin resistance (Neo<sup>r</sup>) gene driven from the MoMuLV long terminal repeat (LTR). The G1XSVNa vector is similar to G1Na but instead contains a multiple cloning site (MCS) downstream of the 5' LTR and expresses the Neo<sup>r</sup> gene from an internal simian virus 40 (SV40) promoter. The GC-SN<sub>DC</sub> vector (Fig. 1B) is identical to G1XSVNa except for the presence of an additional MCS in the 3' LTR. The G1Na,

G1XSVNa, and GC-SN<sub>DC</sub> vectors were derived from the G1 vector (Genetic Therapy, Inc., Gaithersburg, Md.) that has been described earlier (36).

The antisense-TAR retroviral vector GC-SN(anti-TAR)<sub>DC</sub> (Fig. 1C) was constructed as previously described (9). The antisense-TAR sequences were derived from the HIV-1<sub>MN</sub> isolate (20) and were expressed as a tRNA<sup>Met</sup>-antisense-TAR fusion transcript in a double-copy vector design (1, 27, 28, 50). The antisense-Tat/Rev gene used to generate the antisense-Tat/Rev retroviral vector GC-(anti-Tat/Rev)SN<sub>DC</sub> (Fig. 1D) encompassed the complete Rev sequence and the 3' half of the Tat gene, as described previously (6a). The Rev<sup>TD</sup> gene, used to generate the Rev transdominant vectors G1Rev<sup>TD</sup>SN and GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub>, encoded a transdominant mutant version of the HIV-1 Rev protein containing a point mutation (Leu-78 to Asn-78) in the leucine-rich C-terminal domain, which is important for Rev activation (44a). The G1Rev<sup>TD</sup>SN vector was constructed by removing the Rev<sup>TD</sup> gene-containing *Bst*EII-to-*Sfi*I fragment from LR<sup>NSN</sup> (44a) and inserting this fragment into the G1XSVNa vector restricted with the same enzymes. The GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub> vector was constructed by removing the Rev<sup>TD</sup> gene-containing *Bst*EII-to-*Sfi*I fragment from LR<sup>NSN</sup> (44a) and inserting this fragment into the GC-SN(anti-TAR)<sub>DC</sub> vector cut with the same enzymes.

**Transduction of CD4<sup>+</sup> T cells.** The retroviral constructs were transfected by the calcium phosphate method (47) into PA317 (37) and GP+E86 (35) cells, and retroviral vector producer cell lines were obtained by the ping-pong procedure as described previously (6, 9). The titer of the vector-containing supernatant was determined by transducing NIH 3T3 cells (9). Peripheral blood mononuclear cells (PBMC) were obtained from the blood of healthy individuals who had been subjected to apheresis. Erythrocytes were removed from the PBMC fraction by Ficoll-Hypaque (BioWhittaker, Walkersville, Md.) gradient centrifugation. CD4<sup>+</sup> T cells were then enriched by immunomagnetic CD8<sup>+</sup> T-cell depletion with Dynabeads M-450 CD8 (Dynal, Great Neck, N.Y.), seeded at 5 × 10<sup>5</sup> cells per ml in 24-well plates, and expanded in vitro for 2 to 3 days in AIM-V, modification 302 (AIM-V-302) (Gibco, Grand Island, N.Y.), supplemented with 5% fetal calf serum (FCS), 200 U of interleukin 2 (IL-2) per ml, and 10 ng of OKT3 antibodies (Ortho Diagnostic Systems, Raritan, N.J.) per ml. The CD4<sup>+</sup> T cells were subsequently subjected to four consecutive rounds of transductions at 12-h intervals with vector supernatants containing 5 μg of protamine sulfate

per ml, 200 U of IL-2 per ml, and 10 ng of OKT3 per ml. One day after the final transduction, cells were selected by growth in AIM-V-302 containing 0.5 mg of G418 per ml, 200 U of IL-2 per ml, and 5% FCS for 7 days and expanded for an additional 2 days in the absence of G418. Cell viability was determined by trypan blue exclusion from live cells in order to estimate transduction efficiencies. Subsequently, dead cells were removed by Ficoll-Hypaque density gradient centrifugation prior to HIV-1 challenge.

**HIV-1 infection.** Transduced CD4<sup>+</sup> T cells (10<sup>6</sup>/0.5 ml of AIM-V-302 with 200 U of IL-2 per ml and 5% FCS plus 2 µg of Polybrene per ml) were mixed with 0.5 ml of serially diluted HIV-1 supernatant in a single well of a 24-well plate. The cells were incubated overnight with virus at 37°C in a 5% CO<sub>2</sub> atmosphere. The CD4<sup>+</sup> T cells were infected with the primary HIV-1 isolate 301657 (multiplicities of infection [MOI] = 1:550 and 1:2,800) or 302054 (MOI = 1:300 and 1:1,600) and the AZT-resistant primary HIV-1 isolate AO12-G691-2 (MOI = 1:35, 1:180, and 1:500) or 14 AO18-G910-6 (MOI = 1:100, 1:500, and 1:1,500). The 50% tissue culture infective doses (TCID<sub>50</sub>) of the various HIV-1 strains were determined beforehand with MT-2 cells (for AO12-G691-2 and 14 AO18-G910-6) and phytohemagglutinin-stimulated PBMC (for 301657 and 302054). These reagents were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and were provided by Gregory Melchers (301657 and 302054) and Douglas Richman (AO12-G691-2 and 14 AO18-G910-6). After the overnight incubation with HIV-1, 1 ml of complete medium was added to each well containing infected CD4<sup>+</sup> T cells. Aliquots of infected CD4<sup>+</sup> T-cell supernatant were collected at different time points and stored frozen at -70°C for subsequent reverse transcriptase (RT) assay. Samples were diluted if necessary to yield RT levels that fell within the linear range of the assay. The RT levels were quantified by using a Betascope 603 instrument (Betagen, Framingham, Mass.) and expressed in counts per minute after subtraction of background.

## RESULTS

**Construction of anti-HIV retroviral vectors.** To test the efficacy of anti-HIV gene therapy in primary T cells, we have developed four different anti-HIV-1 retroviral vectors aimed at inhibiting Tat and Rev function. The retroviral vectors that were used in this study were designed to express (i) antisense-TAR RNA [GC-SN(anti-TAR)<sub>DC</sub>] (Fig. 1C), (ii) antisense-Tat/Rev RNA [GC-(anti-Tat/Rev)SN<sub>DC</sub>] (Fig. 1D), (iii) Rev<sup>TD</sup> proteins (G1Rev<sup>TD</sup>SN) (Fig. 1E), or (iv) a combination of antisense-TAR RNA plus Rev<sup>TD</sup> proteins [GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub>] (Fig. 1F). In all anti-HIV retroviral vectors we incorporated the neomycin resistance phosphotransferase gene driven by the SV40 promoter-enhancer, which can be used as a selectable marker conferring resistance to the neomycin analog G418. In addition to these four different anti-HIV vectors, we used two control vectors that do not contain any anti-HIV gene but only the Neo<sup>r</sup> gene, driven from either the 5' MoMuLV LTR (for the G1Na vector) (Fig. 1A) or an internal SV40 promoter-enhancer (for the GC-SN<sub>DC</sub> vector) (Fig. 1B).

The GC-SN(anti-TAR)<sub>DC</sub>, GC-(anti-Tat/Rev)SN<sub>DC</sub>, G1Rev<sup>TD</sup>SN, GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub>, G1Na (control), and GC-SN<sub>DC</sub> (control) vector DNAs were transfected into a mixed culture of PA317 and GP+E86 cells, and after amplification of the proviral DNA (6, 9) and selection in G418 and hypoxanthine-aminopterin-thymidine, PA317-derived producer cell lines were obtained. The integrity of the retroviral vector DNA was verified by Southern analysis to exclude the possibility that rearranged vector sequences were present (data not shown). Viral supernatant obtained from confluent PA317 monolayers typically yielded titers of 5 × 10<sup>5</sup> to 9 × 10<sup>5</sup> G418<sup>r</sup> CFU per ml. These vector-containing producer cell supernatants were used for subsequent transductions of peripheral blood lymphocytes (PBL). Expression of antisense-TAR, antisense-Tat/Rev, or Rev<sup>TD</sup> by these retroviral vectors was previously confirmed in functional assays including inhibition of HIV-1 gene expression and replication in both transient and stable transfection systems (6a, 9, 44a).

**Transduction of CD4<sup>+</sup> PBL.** We previously reported that retrovirus-mediated gene transfer into primary lymphocytes from humans and nonhuman primates generally resulted in

low levels (<10%) of gene transfer (12, 13, 40) and that the gene-engineered cells can be enriched for by growth in medium containing G418 to select for the Neo<sup>r</sup> gene. In this report, we sought to improve on these conditions and to specifically transduce CD4<sup>+</sup> T lymphocytes. The retroviral transduction conditions and selection scheme used here (for details, see Materials and Methods) reproducibly resulted in successful gene transfer into lymphocytes with limited loss in postselection culture viability. Furthermore, gene insertion and subsequent G418 selection did not substantially alter the growth characteristics of the transduced cells (data not shown), which is consistent with our previously published results (12).

Using culture and transduction conditions adapted from the ADA-SCID (adenosine deaminase-severe combined immunodeficiency) human gene therapy trial (5), we previously observed a short-term shift in cell phenotype in favor of CD8<sup>+</sup> T cells (40) that likely resulted from a more rapid growth rate of the CD8<sup>+</sup> T cells under these conditions. This is a serious limitation for most anti-HIV gene therapy applications, since the CD4<sup>+</sup> T cells and not CD8<sup>+</sup> T cells require protection from HIV-1 infection. To prevent CD8<sup>+</sup> T-cell expansion, these cells were removed prior to the initiation of T-cell culture by immunomagnetic depletion. Because retroviral vectors do not stably integrate into nondividing cells, the CD4<sup>+</sup>-enriched lymphocytes were cultured under conditions that stimulate T-cell proliferation (growth in medium containing IL-2 and antibody to CD3) for 2 to 3 days prior to retroviral transduction. Following four consecutive rounds of transductions over the next 2 days, the CD4<sup>+</sup> lymphocytes were grown in selective medium for 7 days. We modified the selection procedure by augmenting the G418 concentration to 0.5 mg/ml, which resulted in a more effective elimination of the nontransduced cells. However, it was not possible to completely eliminate the nontransduced cell fraction by further augmenting the G418 concentration without causing significant cell mortality and morbidity (40). The percentage of transduced cells before selection was approximately 20%, and within the viable cell fraction after G418 selection approximately 80% of the remaining viable cells were transduced. PCR amplification of the Neo<sup>r</sup> gene confirmed that the CD4<sup>+</sup> T lymphocytes were stably transduced with the anti-HIV retroviral vectors (data not shown). The viable cell fraction, enriched by Ficoll-Hypaque density centrifugation, was subsequently used for HIV-1 challenge.

**Challenge of transduced primary CD4<sup>+</sup> T lymphocytes with clinical HIV-1 isolates.** In the majority of reports of anti-HIV gene therapy studies published to date, the efficacy of these strategies was assessed by infection of T-cell lines with HIV-1 laboratory strains (2, 7-9, 27, 28, 30, 32, 34, 40, 42, 48, 50, 55). While the protection of the SupT1 T-cell line engineered with the Rev<sup>TD</sup> (44a), antisense-Tat/Rev (6a), or antisense-TAR (9) retroviral vectors seemed promising, it was necessary to demonstrate that transduced primary CD4<sup>+</sup> T lymphocytes could be protected from infection by primary patient isolates of HIV-1. Moreover, in comparison of published reports it has been difficult to evaluate which anti-HIV strategy or vector is most effective because of differences in cell lines, HIV-1 strains, infection procedures, or other experimental parameters. To overcome these limitations we compared the anti-HIV potentials of four different anti-HIV retroviral vectors under physiologically relevant conditions. We transduced CD4<sup>+</sup> T lymphocytes with the vectors GC-SN(anti-TAR)<sub>DC</sub>, GC-(anti-Tat/Rev)SN<sub>DC</sub>, G1Rev<sup>TD</sup>SN, GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub>, and GC-SN<sub>DC</sub> (the control vector) and subjected them to G418 selection. The stably transduced CD4<sup>+</sup> T cells were then challenged with clinical HIV-1 isolates 302054 (Fig. 2) and

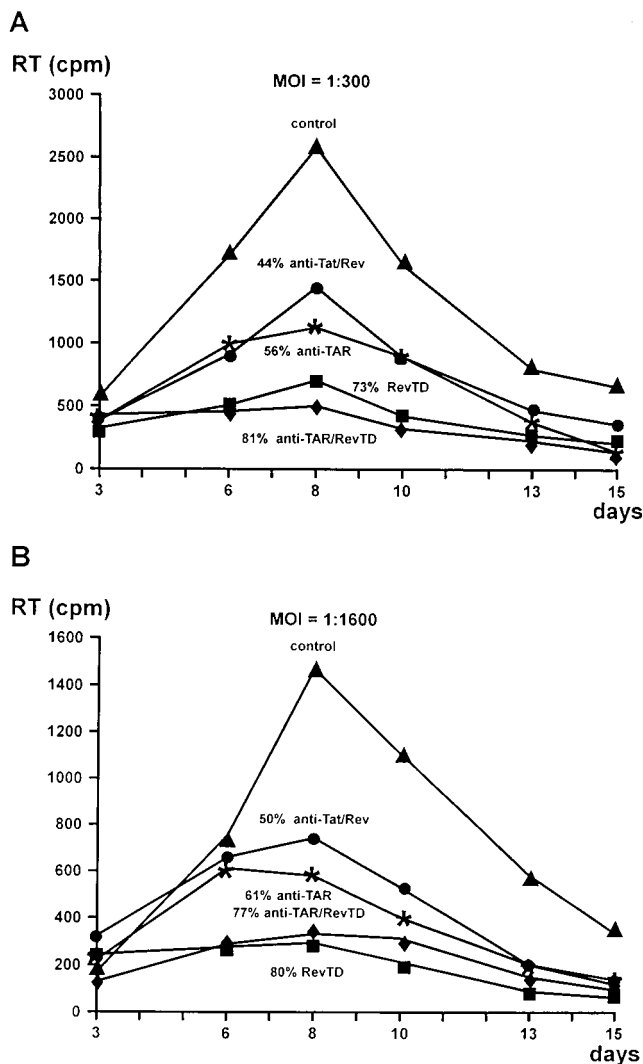


FIG. 2. Comparison of anti-HIV vectors in infected CD4<sup>+</sup> T lymphocytes infected with primary HIV-1 isolate 302054. Shown is RT activity (in counts per minute) by HIV-1 isolate 302054-infected CD4<sup>+</sup> T lymphocytes stably transduced with the control vector GC-SN<sub>DC</sub> (triangle), GC-SN(anti-TAR)<sub>DC</sub> (asterisk), GC-(anti-Tat/Rev)SN<sub>DC</sub> (circle), G1Rev<sup>TD</sup>SN (square), and GC-Rev<sup>TD</sup>. SN(anti-TAR)<sub>DC</sub> (diamond) at MOI of 1:300 (A) and 1:1,600 (B). RT activity was determined by a standard RT assay and quantified by using a Betascope instrument after subtraction of background. The percent inhibition by the anti-HIV vectors in comparison with the control is indicated on the peak of viral production on day 8.

301657 (Fig. 3). Two different amounts of HIV-1 were used for the challenges: for HIV-1 isolate 302054 the MOI used were 1:300 (Fig. 2A) and 1:1,600 (Fig. 2B), and for HIV-1 301657 the MOI were 1:550 (Fig. 3A) and 1:2,800 (Fig. 3B). HIV-1 production was quantified by measuring RT activity.

The CD4<sup>+</sup> T cells transduced with the control vector GC-SN<sub>DC</sub> were readily infected by both primary HIV-1 isolates, as revealed by the rapid increase in RT levels. As expected, increasing the MOI resulted in an increase in RT activity and accelerated viral infection. A decline in RT levels was apparent during the later phase of the infection and may reflect a decrease in cell number due to the cytopathic effects associated with HIV-1 infection. The antisense-TAR, antisense-Tat/Rev, Rev<sup>TD</sup>, and antisense-TAR/Rev<sup>TD</sup> combination vectors inhibited both clinical HIV-1 isolates in the transduced primary

CD4<sup>+</sup> T lymphocytes at both low and high MOI. In all cases, the Rev<sup>TD</sup> vectors inhibited virus replication better (70 to 80% inhibition at the peak of RT production) than did either the antisense-TAR or antisense-Tat/Rev vectors (30 to 60% inhibition at the peak of RT production). Since both the single-gene antisense-TAR and Rev<sup>TD</sup> vectors were both able to individually inhibit HIV-1, we tested whether the inclusion of the antisense-TAR gene into the Rev<sup>TD</sup> vector would further augment its inhibitory potential. The data indicated that the combination vector GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub> was at least as potent (70 to 80% inhibition) as the single anti-HIV gene vector was under the conditions tested and might even be slightly better at inhibiting the primary HIV-1 isolates at higher MOI (Fig. 2A and 3A).

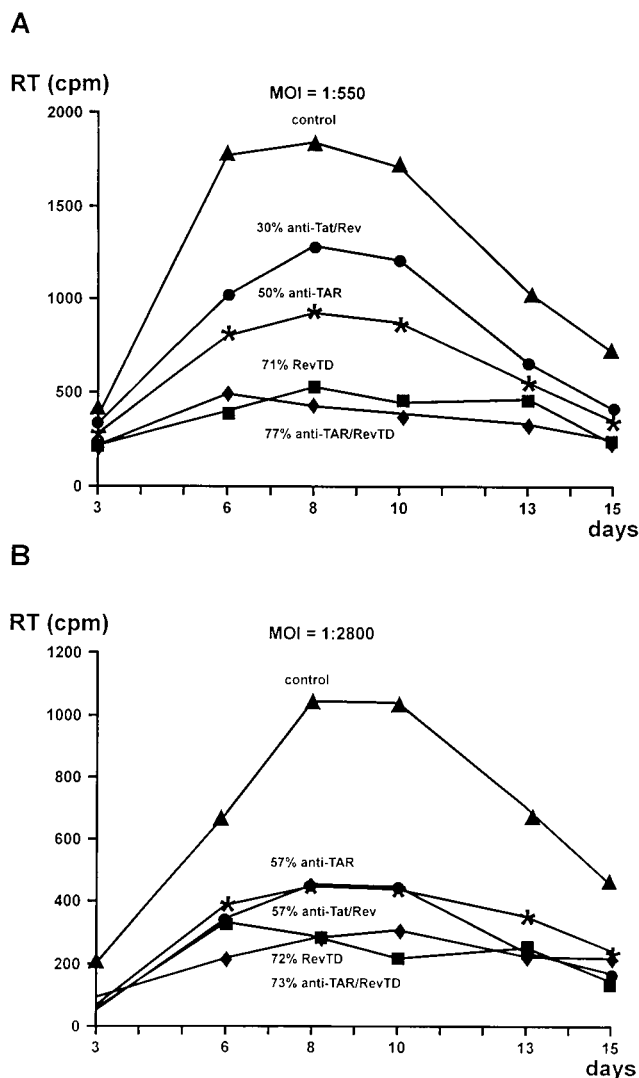


FIG. 3. Comparison of anti-HIV vectors in infected CD4<sup>+</sup> T lymphocytes infected with primary HIV-1 isolate 301657. Shown is RT activity (in counts per minute) by HIV-1 isolate 301657-infected CD4<sup>+</sup> T lymphocytes stably transduced with the control vector GC-SN<sub>DC</sub> (triangle), GC-SN(anti-TAR)<sub>DC</sub> (asterisk), GC-(anti-Tat/Rev)SN<sub>DC</sub> (circle), G1Rev<sup>TD</sup>SN (square), and GC-Rev<sup>TD</sup>. SN(anti-TAR)<sub>DC</sub> (diamond) at MOI of 1:550 (A) and 1:2,800 (B). RT activity was determined by a standard RT assay and quantified by using a Betascope instrument after subtraction of background. The percent inhibition by the anti-HIV vectors in comparison with the control is indicated on the peak of viral production on day 8.

AZT-resistant HIV-1 variants frequently arise in HIV-1-infected individuals who are subjected to AZT therapy (45). Since the emergence of drug-resistant strains leads to diminished effectiveness of the drug, it is important to evaluate if gene therapy may be an effective alternative to suppress these resistant strains. We therefore assessed if AZT-resistant clinical isolates can be inhibited with anti-HIV retroviral vectors. We also wanted to evaluate if the differences in anti-HIV potential among the various anti-HIV vectors mentioned above could be corroborated by using these additional primary HIV-1 isolates. We transduced CD4<sup>+</sup> T lymphocytes with either GC-SN(anti-TAR)<sub>DC</sub>, GC-(anti-Tat/Rev)SN<sub>DC</sub>, G1Rev<sup>TD</sup>SN, GCRev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub>, or the GC-SN<sub>DC</sub> control vector and subsequently challenged these cells with two unrelated AZT-resistant clinical HIV-1 isolates, AO12-G691-2 (Fig. 4) and 14 AO18-G910-6 (Fig. 5). Three different infectious doses of virus were used in an attempt to titrate the inhibitory potential of the anti-HIV vectors. For the AO12-G691-2 isolate, the MOI used were 1:35 (Fig. 4A), 1:180 (Fig. 4B), and 1:500 (Fig. 4C). The MOI for the other isolate, 14 AO18-G910-6, were 1:100 (Fig. 5A), 1:500 (Fig. 5B), and 1:1,500 (Fig. 5C). The kinetics of replication of the HIV-1 AZT-resistant isolates, again monitored by RT activity, were similar to the properties of the primary strains 302054 (Fig. 2) and 301657 (Fig. 3), described above. Viral production increased rapidly with increasing MOI and consistently declined in the later phase of HIV-1 infection. All anti-HIV retroviral vectors tested inhibited the two AZT-resistant clinical HIV-1 isolates in the transduced primary CD4<sup>+</sup> T lymphocytes at both low and high MOI, as was observed with primary isolates 302054 (Fig. 2) and 301657 (Fig. 3). The relative differences in the inhibitory potential of the various anti-HIV retroviral vectors were also reproducible since the Rev<sup>TD</sup> vectors inhibited these clinical isolates more potently (50 to 80% inhibition at the peak of RT production) than did either the antisense-TAR or antisense-Tat/Rev vectors (20 to 50% inhibition at the peak of RT production). Finally, using these AZT-resistant isolates we also confirmed that the Rev<sup>TD</sup>/antisense-TAR combination vector is at least as potent as the Rev<sup>TD</sup> single-gene vector in the given MOI range (70 to 80% inhibition) and is sometimes even slightly better (Fig. 4B and 5A).

## DISCUSSION

We have demonstrated here that HIV-1 clinical isolates were inhibited effectively in primary CD4<sup>+</sup> T lymphocytes by retroviral vectors expressing antisense-TAR, antisense-Tat/Rev, and Rev<sup>TD</sup> proteins. We further showed that inhibition by Rev<sup>TD</sup> was more potent than was inhibition by the antisense vectors in transduced CD4<sup>+</sup> T lymphocytes. A plausible explanation for this difference was that only a few Rev<sup>TD</sup> molecules were sufficient to incorporate into multimeric complexes with multiple wild-type Rev molecules (22). Consequently, the incorporation of just a few Rev<sup>TD</sup> monomers into this higher-order structure could simultaneously disrupt the function of many wild-type Rev monomers, making this strategy highly effective in inhibiting HIV-1. In contrast, the antisense-TAR and antisense-Tat/Rev molecules probably inhibited HIV-1 gene expression through the formation of RNA-RNA duplexes that triggered degradation or prevented subsequent translation. Such a mechanism is consistent with the previously published observations showing that accumulation of TAR-containing mRNA and Tat/Rev mRNA was inhibited by the corresponding antisense vectors (8, 49). This mechanism also implies that one antisense RNA molecule can inactivate only one complementary target sequence. This 1:1 stoichiometry

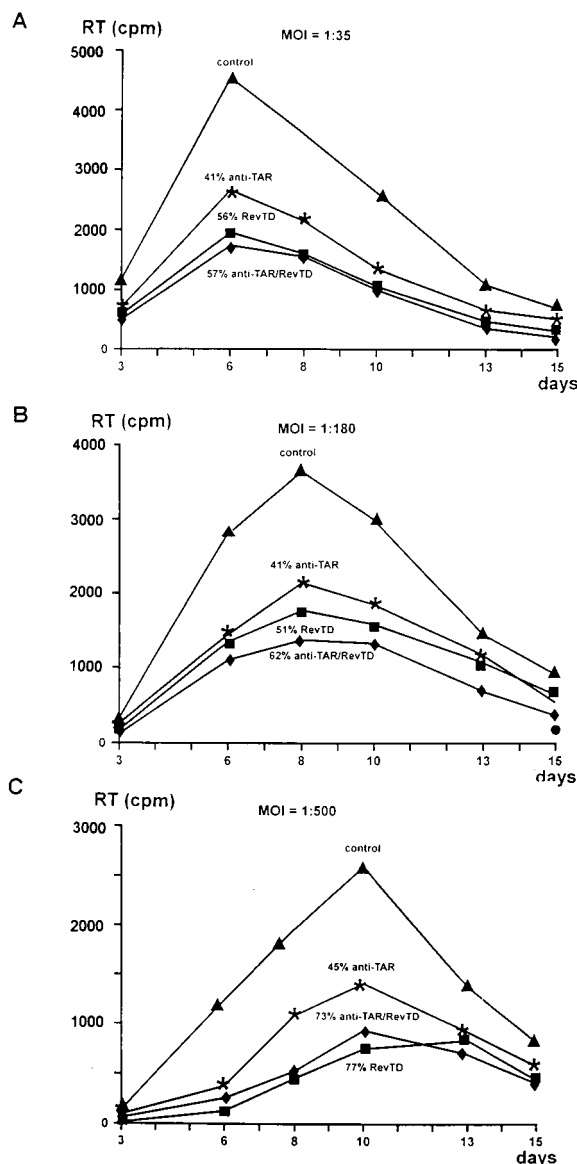


FIG. 4. Comparison of anti-HIV vectors in infected CD4<sup>+</sup> T lymphocytes infected with AZT-resistant primary HIV-1 isolate AO12-G691-2. Shown is RT activity (in counts per minute) by HIV-1 isolate AO12-G691-2-infected CD4<sup>+</sup> T lymphocytes stably transduced with the control vector GC-SN<sub>DC</sub> (triangle), GC-SN(anti-TAR)<sub>DC</sub> (asterisk), G1Rev<sup>TD</sup>SN (square), and GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub> (diamond) at MOI of 1:35 (A), 1:180 (B), and 1:500 (C). RT activity was determined by a standard RT assay and quantified by using a Betascope instrument after subtraction of background. The percent inhibition by the anti-HIV vectors in comparison with the control is indicated on the peak of viral production on day 6 (A), day 8 (B), and day 10 (C).

may account for the diminished effectiveness of the antisense RNA compared with the transdominant protein strategy. However, other mechanisms may also contribute to the better inhibition seen with Rev<sup>TD</sup>, such as higher expression levels, increased stability, and a more favorable intracellular localization.

In most PBL challenge experiments, we achieved almost 80% protection with our most potent Rev<sup>TD</sup> vectors (Fig. 2–4). The viability data that we obtained after G418 selection suggest that approximately 80% of the viable CD4<sup>+</sup> cells used in the challenge experiments were transduced. Since almost 80%

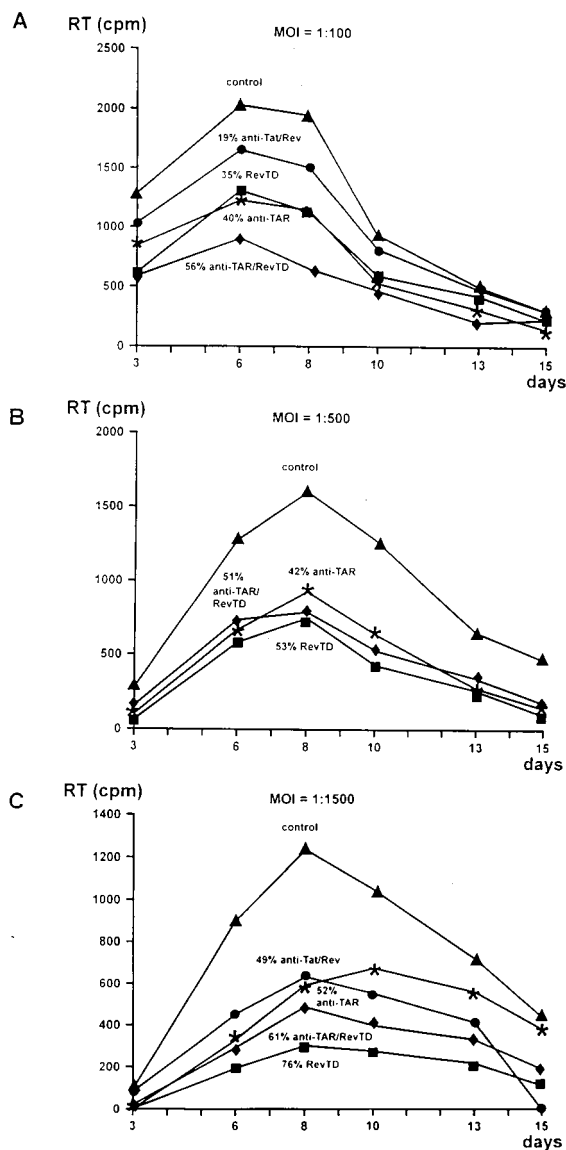


FIG. 5. Comparison of anti-HIV vectors in infected CD4<sup>+</sup> T lymphocytes infected with AZT-resistant primary HIV-1 isolate 14 AO18-G910-6. Shown is RT activity (in counts per minute) by HIV-1 isolate 14 AO18-G910-6-infected CD4<sup>+</sup> T lymphocytes stably transduced with the control vector GC-SN<sub>DC</sub> (triangle), GC-SN(anti-TAR)<sub>DC</sub> (asterisk), GC-(anti-Tat/Rev)SN<sub>DC</sub> (circle), G1Rev<sup>TD</sup>SN (square), and GC-Rev<sup>TD</sup>SN(anti-TAR)<sub>DC</sub> (diamond) at MOI of 1:100 (A), 1:500 (B), and 1:1,500 (C). RT activity was determined by a standard RT assay and quantified by using a Betascope instrument after subtraction of background. The percent inhibition by the anti-HIV vectors in comparison with the control is indicated on the peak of viral production on day 6 (A) and day 8 (B and C).

protection had been achieved with the Rev<sup>TD</sup> vectors, this suggests that the majority of all the transduced cells were protected and that the observed viral production is most likely due to the residual nontransduced cell fraction, which constitutes 20% of the viable cell fraction. In the most stringent conditions, at high MOI (Fig. 4), about 60% protection could still be achieved in CD4<sup>+</sup> T lymphocytes engineered with the Rev<sup>TD</sup> or Rev<sup>TD</sup>/anti-TAR vector. These data indicated that Rev<sup>TD</sup> proteins were potent inhibitors of HIV-1. However, the question remains as to which in vitro challenge dose of virus mimics the true in vivo condition, which varies greatly depend-

ing on the clinical stage and anatomic location (14, 44). The level of expression of the Rev<sup>TD</sup> protein by the retroviral vector may be sufficiently high to protect engineered cells from HIV-1 infection in vivo when cells are exposed to the typically low levels of HIV-1 particles in the circulation. In contrast, since HIV-1 infection is active and progressive in the lymph nodes during the clinically latent stage of the disease, it is likely that the engineered cells, when administered in vivo, would be confronted with a large number of HIV-1-infected cells in the lymphoid tissues. However, this does not necessarily imply that the engineered cells would be exposed to a high dose of replication-competent HIV-1 particles. The high rate of errors in retrovirus replication could result in many defective HIV-1 quasispecies, as shown for HIV-1 isolates directly cloned from uncultured human brain tissue (29). If so, cells infected by defective viruses might not be killed by virus replication and might in some cases even become resistant to superinfection by nondefective viruses (51). Furthermore, a large proportion of the cells in the germinal centers of the lymph nodes are latently infected with HIV-1, since they do not express any viral RNA despite the presence of an integrated HIV-1 provirus as revealed by double-label in situ PCR (14).

The HIV-1 challenge experiments were performed at various MOI, to determine if protection against HIV-1 persisted when the amount of input virus was increased. Our studies consistently revealed that inhibition of HIV-1 production was decreased when the transduced CD4<sup>+</sup> T lymphocytes were challenged with a higher dose of HIV-1 virus. This is consistent with the observations of others that anti-HIV gene therapy strategies could not prevent viral breakthrough in T-cell lines that were challenged with a relatively high viral dose (2, 9, 34, 49, 50). It has been suggested that because of the inherent clonal variation in expression levels of the anti-HIV genes in the transduced cell population, it is possible that some of the transduced cells (those that express lower levels of the anti-HIV genes) may not be protected when challenged with a higher dose of HIV-1. Moreover, since the viable CD4<sup>+</sup> lymphocyte fraction, which was subjected to HIV-1 challenge, consisted of approximately 80% G418<sup>r</sup> transduced cells and 20% background nontransduced cells, this residual nontransduced lymphocyte fraction may readily become infected with HIV-1, resulting in decreased inhibition at higher MOI. Alternatively, the possibility that the decreased inhibition at high MOI may be due to the emergence of escape mutants in vitro cannot be excluded a priori, although this was not observed to be the case for our Rev<sup>TD</sup> mutants (44a).

Since both the antisense-TAR and Rev<sup>TD</sup> single anti-HIV gene retroviral vectors effectively inhibited the HIV-1 clinical isolates, we wanted to test if inclusion of multiple anti-HIV genes into the same retroviral vector backbone may further increase its anti-HIV potency. We showed that the Rev<sup>TD</sup>/antisense-TAR combination vector is at least as effective as the Rev<sup>TD</sup> single-gene vector at inhibiting the primary HIV-1 isolates. In some cases, especially at high MOI (e.g., see Fig. 2A, 3A, 4B, and 5A), the combination vector is slightly better than the Rev<sup>TD</sup> single-gene vector. Hence, the inclusion of the antisense-TAR gene into the G1Rev<sup>TD</sup>SN retroviral vector may slightly improve its inhibitory potential under the conditions tested. This suggests that HIV-1 replication can efficiently be inhibited in PBL that express the transdominant protein and that the addition of a second anti-HIV gene, such as the antisense-TAR gene, may be beneficial when the cells are exposed to an excessive amount of HIV-1. One additional advantage of combination vectors containing multiple anti-HIV genes, such as GC-Rev<sup>TD</sup>-SN(anti-TAR)<sub>DC</sub>, is that escape mutants which simultaneously acquire resistance to both anti-HIV genes are

less likely to emerge. In view of these advantages, it is necessary to further develop and compare vectors expressing various combinations of known anti-HIV genes.

Our data on the inhibition of HIV-1 by Rev<sup>TD</sup> are consistent with the observations of Malim and coworkers and other investigators, who showed that a distinct transdominant Rev mutant protein (M10) containing two adjacent point mutations in the activator domain (Leu-78 to Asp-78 and Glu-79 to Leu-79) inhibited HIV-1 in COS cells (32) and on stable retrovirus-mediated transduction of the human T-cell line CEM (2, 30, 34). Our data further showed that the transdominant Rev phenotype can be conferred by a single point mutation in the activator domain (Leu-78 to Asn-78) (44a). Decreased HIV-1 production by antisense-TAR and antisense-Tat/Rev vectors has also been demonstrated previously by transduction of the human A3.01 T-cell line with an antisense-TAR adeno-associated virus vector (8) and by stable transfection of an antisense-Tat/Rev expression plasmid in Jurkat and SupT1 cells (6a, 49). Although the results of these earlier studies seemed promising, it was necessary to assess whether effective inhibition of HIV-1 by either Rev<sup>TD</sup> or the antisense-TAR or antisense-Tat/Rev vector occurred under conditions relevant for clinical gene therapy, such as with primary CD4<sup>+</sup> T lymphocytes instead of T-cell lines and with challenge by clinical HIV-1 isolates. Similar results were obtained with the AZT-sensitive and -resistant viruses. This could be expected since AZT resistance results from mutations in the Pol gene (45) that do not affect the targets of our gene therapy strategies, which are aimed at disrupting Tat and Rev function. Our current studies demonstrate that human primary CD4<sup>+</sup> T lymphocytes can be protected by antisense RNA or transdominant mutant proteins against infection with clinical HIV-1 isolates, including AZT-resistant strains. These data complement the recent findings of Leavitt and coworkers (26), who showed that expression by a retroviral vector of a hairpin ribozyme targeted at the HIV-1 leader sequence could also inhibit the replication of clinical HIV-1 isolates in primary CD4<sup>+</sup> T cells. It remains to be seen which approach is more effective at inhibiting HIV-1.

In contrast to therapy with conventional drugs, such as RT inhibitors, which have no effect on HIV-1 gene expression from integrated proviruses, gene therapy may be a clinically applicable method for the specific down-regulation of HIV-1 gene expression. This is important in view of the notion that certain HIV-1 gene products may be associated with virus-independent pathological manifestations. For example, in addition to acting on HIV-1 itself, the Tat protein may also contribute to AIDS-associated Kaposi's sarcoma (15), B-cell lymphomas (25), and neuropathologies (46). The Rev-dependent expression of the HIV-1 envelope protein gp120 has also been shown to lead to pathological manifestations independent of HIV-1 replication, such as abnormal lymphocyte proliferation (38), activation-induced apoptosis (3, 31), and potential neuronal damage (21). The specific inhibition of Tat and Rev expression by gene therapy using intracellularly expressed antisense or transdominant proteins may attenuate these clinical manifestations associated with AIDS.

Since inhibiting the production of clinical HIV-1 isolates in normal human CD4<sup>+</sup> T lymphocytes, the major primary target cells for HIV-1 infection, is feasible, it will now be crucial to evaluate the efficacy of these anti-HIV-1 gene therapy strategies in HIV-1-infected individuals. It may be possible to use HIV-1-discordant identical twins to simultaneously evaluate the effectiveness of several different anti-HIV-1 retroviral vectors simultaneously administered to the infected patient (54). The availability of healthy CD4<sup>+</sup> T lymphocytes from the uninfected twin permits rigorous expansion and transduction with

anti-HIV vectors under conditions that may be difficult with CD4<sup>+</sup> T lymphocytes from the infected twin. These uninfected engineered CD4<sup>+</sup> T lymphocytes can then be returned to the infected twin to determine which gene therapy approach provides the most effective protection to gene-modified CD4<sup>+</sup> T lymphocytes *in vivo*. Only clinical trials will be able to address the question of whether rendering a mature CD4<sup>+</sup> T lymphocyte resistant to HIV-1 infection by blocking HIV-1 gene expression will have therapeutic benefit to patients, particularly late in the course of AIDS progression.

#### ACKNOWLEDGMENTS

M.K.L.C. and T.V. contributed equally to this work.

We thank R. Michael Blaese and Robert C. Gallo for their continuous support, and Anthony Fauci is acknowledged for providing HIV-1 challenge facilities. We also thank Theresa Lumsden for secretarial assistance.

T.V. is a Research Fellow of the Belgian National Fund for Scientific Research (Nationaal Fonds voor Wetenschappelijk Onderzoek, Kom Op Tegen Kanker Actie).

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