

An Autoregulated Dual-Function *antit* Gene for Human Immunodeficiency Virus Type 1 Gene Therapy

JULIANNA LISZIEWICZ,^{1*} DAISY SUN,¹ BRUCE TRAPNELL,² MICHAEL THOMSON,¹
HSIAO-KUEY CHANG,¹ BARBARA ENSOLI,¹ AND BO PENG²

Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Maryland 20892,¹
and Genetic Therapy Incorporated, Gaithersburg, Maryland 20878²

Received 5 July 1994/Accepted 5 October 1994

One approach to gene therapy for AIDS is to block the replication of human immunodeficiency virus type 1 (HIV-1) by inhibiting the *tat* gene, whose product activates the expression of all HIV-1 genes. To accomplish this, we constructed an *antit* gene expressing an RNA with dual (polymeric TAR and antisense-*tat*) function in an attempt to both sequester Tat protein and block its translation from mRNA. A minigene consisting of the *antit* gene driven by the HIV-1 long terminal repeat was inserted into a double-copy retrovirus vector, such that *antit* expression would be upregulated only in HIV-1-infected cells. After transduction of a T-lymphocytic cell line (Molt-3) the *antit* gene inhibited HIV-1 replication. This inhibition was inversely correlated with the virus infectious dose. Virus replication was also inhibited for 5 months in two different T-cell lines after they had been infected at a high multiplicity of infection, suggesting that the *antit* gene may be effective over long periods. Importantly, *antit* blocked the replication and the cytopathic effect of HIV-1 in human peripheral blood mononuclear cells and led to as much as 4,000-fold inhibition of the replication of an HIV-1 field isolate as well as HIV-1 prototypes maintained in culture. These results suggest that *antit* gene therapy has potential use for blocking HIV-1 replication in infected individuals.

One therapeutic strategy for human immunodeficiency virus type 1 (HIV-1)-infected individuals is to reconstitute the immune system autologously with cells which are rendered nonpermissive for HIV-1 replication. Gene therapy could provide one approach to this goal by stably transducing target cells of HIV-1 infection with a vector expressing an inhibitory gene that is able to block HIV-1 replication. To be effective, such an inhibitory gene should be integrated for long-term effects and express the inhibitory product in all HIV-1-infected cells. Key factors for the success of this strategy include approaches which prevent the formation of escape mutants or viral resistance, a major problem in current HIV-1 treatments. For example, escape mutants should be less likely to occur if the inhibitory molecule could confer multiple constraints on HIV-1 in such a way that escape from therapy would require multiple simultaneous mutations. In addition, the expression of the inhibitory gene should be regulated, like all cellular genes, to avoid potential problems caused by constitutive long-term expression; after integration, the protective gene should not significantly alter cell viability or function.

The *tat* gene product of HIV-1 is a key target for gene therapy not only because it is an early transactivator of the expression of all HIV-1 genes (1, 10, 33) but also because it may play an important role in the pathogenesis of AIDS and associated malignancies as both an extracellular protein and an activator of cellular gene expression (4, 12–15). Another reason to choose *tat* as a target for HIV-1 gene therapy is because portions of the primary and secondary sequences of the Tat activation response element (TAR) must be maintained for correct function. Thus, there are further constraints on *tat* in terms of its own primary, secondary, and tertiary structures to ensure the correct *tat*-TAR binding necessary for

HIV-1 replication. This suggests that mutational escape from a therapy which sequesters *tat* through delivery of TAR decoys requires simultaneous *tat* and TAR mutations in such a way as to not only preserve the interaction of these two molecules but also maintain the ability to activate HIV-1 replication.

We have previously shown that cotransfection of two *tat*-inhibitory genes, polymeric TAR and antisense-*tat* (AS-TAT), has additive effects in blocking HIV-1 gene expression (9). To further develop this strategy for HIV-1 gene therapy, a new inhibitory gene was constructed to express a unique, dual-function inhibitory RNA molecule. This RNA interferes with Tat by inhibiting the translation of the *tat* mRNA (by an antisense mechanism) and by blocking the function of Tat which has escaped from the antisense protection (by polymeric TAR, which sequesters the Tat protein). We report here that in the presence of autoregulation (activation of the transcription of the *tat*-inhibitory gene by the Tat protein from HIV-1), which does not allow overexpression of the inhibitory RNA, this dual-function inhibitory RNA successfully inhibits virus replication in both immortalized T cells and primary peripheral blood-derived human T lymphocytes.

MATERIALS AND METHODS

Molecular cloning. The anti-*tat* gene (Fig. 1A) was constructed by ligation of the HIV-1 long terminal repeat (LTR)-25STAR (21) and the AS-TAT II gene (designated as AS-TAT) described previously (9): pLTR25STAR was digested with *Bam*HI, blunt ended, and ligated with the blunt-ended AS-TAT DNA fragment (*Pst*I). The orientation of the AS-TAT DNA fragment was determined by restriction enzyme digestions. The LTR-25STAR-AS-TAT cassette was then placed into the 3' LTR of a double-copy retrovirus vector (G3) (Genetic Therapy, Inc., Gaithersburg, Md.) in both orientations by blunt-end ligation. G3 is derived from the G1 retrovirus vector (24) by insertion of a multiple-cloning site into the U3 region of the 3' LTR as described by Hantzopoulos et al. (17). The structure of the retrovirus vectors containing the *antit* gene (Fig. 1B) was confirmed by restriction enzyme analysis.

Transient gene transfer of inhibitory plasmids. To demonstrate inhibition of Tat transactivation, COS cells were transfected with different plasmids by liposome-mediated gene transfer with Lipofectin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as specified by the manufacturer. LTR-CAT plasmid (2 µg) was used as a reporter of HIV-1 gene expression, and LTR-TAT

* Corresponding author. Mailing address: Laboratory of Tumor Cell Biology, Bldg. 37, Rm. 6A09, National Cancer Institute, 37 Convent Dr. MSC 4255, Bethesda, MD 20892-4255. Phone: (301) 496-1234. Fax: (301) 496-8394.

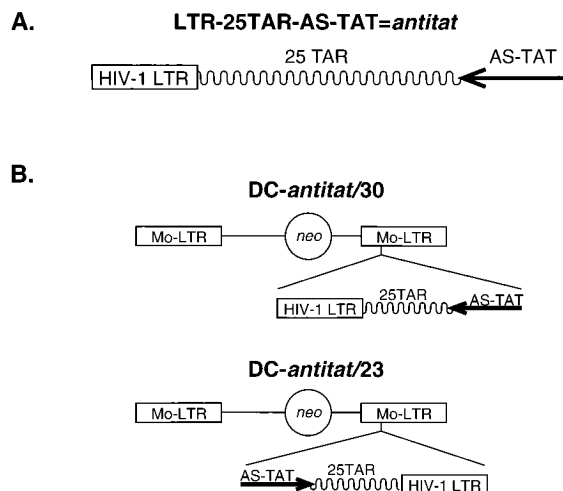


FIG. 1. (A) Structure of the *antitap* inhibitory gene. (B) Retrovirus vectors containing the autoregulated *antitap* inhibitory gene. Double-copy retrovirus vector (17) was used to insert two copies of the *antitap* gene into the cells.

plasmid (0.7 μ g) was used as the transactivator (20). Samples (1 μ g) of the different inhibitory or control plasmids were also cotransfected into these cells. Chloramphenicol acetyltransferase (CAT) activity was determined as described previously (28).

Production of amphotropic retrovirus vectors. To avoid deletions or rearrangements during packaging, plasmids containing the *antitap* retrovirus vector (Fig. 1B) were linearized (with *Nde*I) to permit integration outside of the retrovirus vector cassette. Linearized plasmids were then transfected into the PA317 amphotropic packaging cell line (26) and selected with G418 (500 μ g/ml, active drug). Resistant colonies were pooled, and supernatants were collected for gene transfer experiments. The intactness of the inhibitory gene in the retrovirus vectors was verified by Southern blot experiments.

Gene transfer and HIV-1 infection of immortalized T cells. Molt-3 cells, cultured in RPMI medium supplemented with 15% fetal calf serum, were transfected with medium from vector-producing cells, and G418 (1 mg/ml, active drug)-resistant cell populations were selected without single-cell cloning. These populations were challenged with HIV-1_{HTLV-III-B} (multiplicity of infection [MOI], 0.01 to 1). Supernatants were collected twice a week, and cells were split into portions containing 0.5×10^6 cells per ml. Virus replication was monitored by p24 antigen capture assay (Coulter, Hialeah, Fla.), and the number of viable cells was determined as described by Pauwels et al. (30).

Gene transfer and infection of primary lymphocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of normal donors by Ficoll-Hypaque density gradient centrifugation, activated with phytohemagglutinin (Difco, Detroit, Mich.), and cultured in RPMI medium supplemented with 15% fetal calf serum and 100 U of interleukin-2 (Boehringer Mannheim Biochemicals) per ml. After 2 days, aliquots of PBMCs (10^6 cells per ml) were transfected repeatedly with medium from vector-producing cells and selected with G418 (250 or 500 μ g/ml, active drug) for 10 days. Following recovery (2 days), cells were infected with HIV-1. Supernatants were collected twice a week, and cells were split into portions containing 10^6 cells per ml. HIV-1 replication was monitored by p24 antigen capture assay (Coulter), and viable-cell numbers were assessed as described by Pauwels et al. (30).

Determination of the T-cell phenotype by flow-cytometric analysis. To quantify the depletion of CD4⁺ T cells after HIV-1 infection, PBMCs were washed and treated with fluorochrome-labeled monoclonal antibodies directed against CD4 and CD8 antigens (Becton Dickinson, San Jose, Calif.) at 2 μ g/ml. The cells were washed again, fixed with 2% paraformaldehyde, and analyzed in a flow cytometer (Becton Dickinson).

PCR analysis. To verify the enrichment through G418 selection of transduced PBMCs, genomic DNA was isolated from the transduced cells before and after G418 selection. PCR standards were prepared by mixing (0 to 30%) genomic DNA isolated from the G1 vector producer (PA317) cell line which contains one copy of the neomycin phosphotransferase gene per cell with genomic DNA isolated from the PA317 cell line. Standard PCRs were carried out in Perkin-Elmer Cetus equipment with primers complementary to the *neo* gene (5'-GGTG GAGAGCTATTCGGCTATGA and 5'-ATCCTGATCGACAAGACCGGCTTC).

RESULTS

Mode of action of the *antitap* gene. A heterologous minigene containing a modified HIV-1 LTR (20) followed by 25 repeats

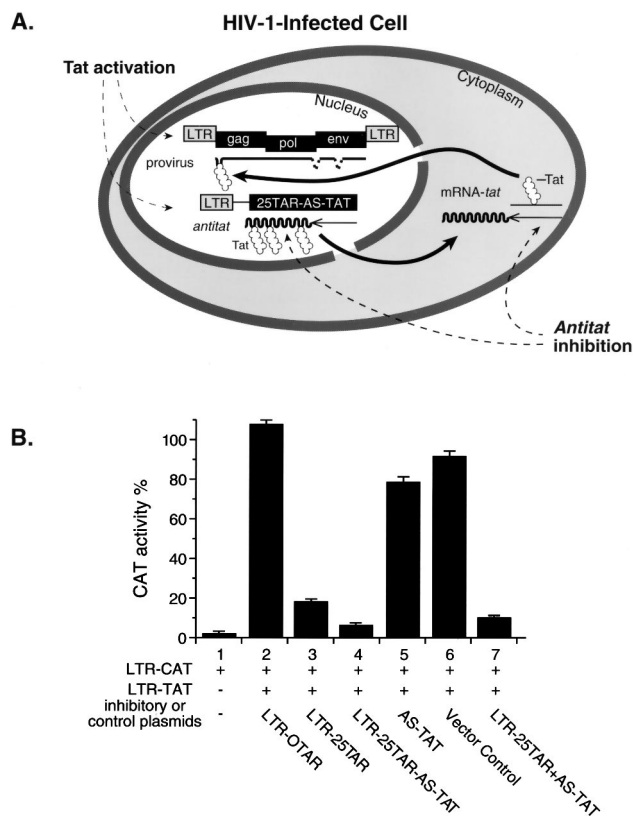


FIG. 2. (A) Mechanism of inhibition of HIV-1 replication by the *antitap* gene. The *antitap* inhibitory product is an RNA molecule which has dual functions: (i) it sequesters the Tat protein by the expression of the polymeric-TAR decoy, and (ii) it blocks the translation of the *tat* mRNA by the antisense component; both of these functions result in inhibition of gene expression, which would otherwise be induced by Tat. *antitap* is under autocrine regulation, because transcriptional activation of the inhibitory molecule is dependent on the amount of the Tat protein. Thus, there is no overexpression of the *antitap* gene even in HIV-1-infected cells. (B) Combination of two *tat*-inhibitory genes in one transcript has an additive effect in the inhibition of Tat function. COS cells were cotransfected with the indicated plasmids. LTR-CAT was used as a reporter of HIV-1 gene expression, and LTR-TAT was used as the transactivator. Inhibition is expressed as percent CAT activity of the positive control LTR-0TAR (LTR without the inhibitory TAR sequence).

of the TAR sequence contiguous with an AS-TAT molecule was constructed (9) (Fig. 1A). The expression of this construct, designated *antitap*, is dependent on Tat activation since it is driven by the HIV-1 LTR (1, 20, 33). Thus, the *antitap* gene should be expressed only in cells containing the Tat protein. The mode of action is illustrated in Fig. 2A. Tat produced by the provirus or taken up by the cell from the extracellular fluid (13, 15) activates transcription of both viral and *antitap* RNA. *antitap* RNA, on the other hand, decreases the amount of Tat protein, resulting in less proviral activation and also downregulation of the *antitap* gene. This activation and inhibition leads to an equilibrium between Tat and *antitap*; consequently, neither Tat nor *antitap* can be overexpressed (autocrine regulation).

antitap uses two different mechanisms to inhibit Tat function: the polymeric TAR RNA can bind and sequester the Tat protein in the nucleus (21), and the AS-TAT RNA can inhibit the translation of the *tat* mRNA in the cytoplasm (9). We have previously shown that polymeric TAR can almost completely block Tat-induced activation of HIV-1 gene expression (21). Therefore, we used suboptimal conditions (no molar excess of

the inhibitory gene) in transient gene transfer experiments to demonstrate the combined efficacy of the two *tat*-inhibitory genes, LTR-25TAR and AS-TAT. Figure 2B shows that LTR-25TAR inhibits 81% of Tat transactivation, AS-TAT inhibits 23%, and the dual-function *antitatt* gene (lane 4) inhibits 94%. A similar result (90% inhibition) was obtained by cotransfection of the LTR-25TAR plasmid with the AS-TAT plasmid (lane 7), indicating that the combination of two *tat*-inhibitory genes has an additive effect in blocking HIV-1 gene expression (9). Our experiments further demonstrate that the activity of the anti-*tat* gene depends on both inhibitory mechanisms, i.e., the polymeric TAR and the AS-TAT RNA components.

Short-term inhibition of HIV-1 replication. Replication-defective retrovirus vectors have already been used in experimental clinical trials to deliver and stably integrate a foreign gene into human cells (reviewed 27). Therefore, the *antitatt* gene was inserted into a Moloney murine leukemia virus-based vector, G3 (17, 24), with the insert in both forward and reverse orientations. These constructs (Fig. 1B) were stably transfected into the amphotropic packaging line, PA317. Southern blot analysis of transfected-cell DNA demonstrated the preservation of the structure of the introduced plasmids. Medium from vector-producing cells was collected to assess the efficacy of the *antitatt* gene in both immortalized and primary lymphocytes.

The anti-HIV-1 activity of the *antitatt* gene was initially evaluated by transduction of a CD4⁺ T-cell line, Molt-3. G418-resistant cell populations were obtained without single-cell cloning and challenged with HIV-1_{HTLV-III_B} at various MOIs. At a very high MOI of 1, HIV-1 replication was blocked for up to 7 days (Fig. 3A). At this MOI, no differences were seen in the forward- and reverse-oriented anti-*tat* gene vectors. In contrast, the G1 vector (not containing the *antitatt* gene) used as a control did not show any antiviral activity. In parallel experiments, as the MOI was decreased 10- and 100-fold (Fig. 3B and C), the time frame of complete inhibition of viral spread increased up to 40 days for the forward vector (DC-*antitatt*/30) and 14 days for the reverse orientation vector (DC-*antitatt*/23).

Long-term inhibition of HIV-1 replication. One advantage of this gene therapy strategy is that the inhibitory gene is integrated and can potentially prevent virus replication permanently. The long-term antiviral effect of the *antitatt* gene in transduced Molt-3 cells infected with HIV-1_{HTLV-III_B} at a high MOI of 1 was evaluated. Importantly, antiviral activity persisted for 5 months (Fig. 4A). Although cells transduced by both *antitatt* and the control vectors showed p24 antigen production shortly after HIV-1 infection because of the higher MOI, the levels were 20- to 30-fold lower in *antitatt*-transduced cells than in cells transduced with the control vector. The viability of the protected cells was comparable to that of the uninfected ones, in contrast to the massive syncytium formation and death of the HIV-1-infected control cells. For example, 5 months after HIV-1 infection, *antitatt*-transduced cells were alive and maintained a normal histologic appearance. At this time, very low but detectable levels of p24 antigen were measured, which is consistent with the expected autocrine control of *antitatt* gene expression. This indicates that the regulatory interactions between Tat and *antitatt* are sufficient to control virus replication for several months in Molt-3 cells even after a high challenge dose of virus.

Comparable results were obtained with another CD4⁺ T-cell line, CEM-SS (Fig. 4B); however, in this case, cells transduced with the control vector were not killed after a high-MOI challenge. In the initial phase of the infection there was only about 70% inhibition of HIV-1 replication; however,

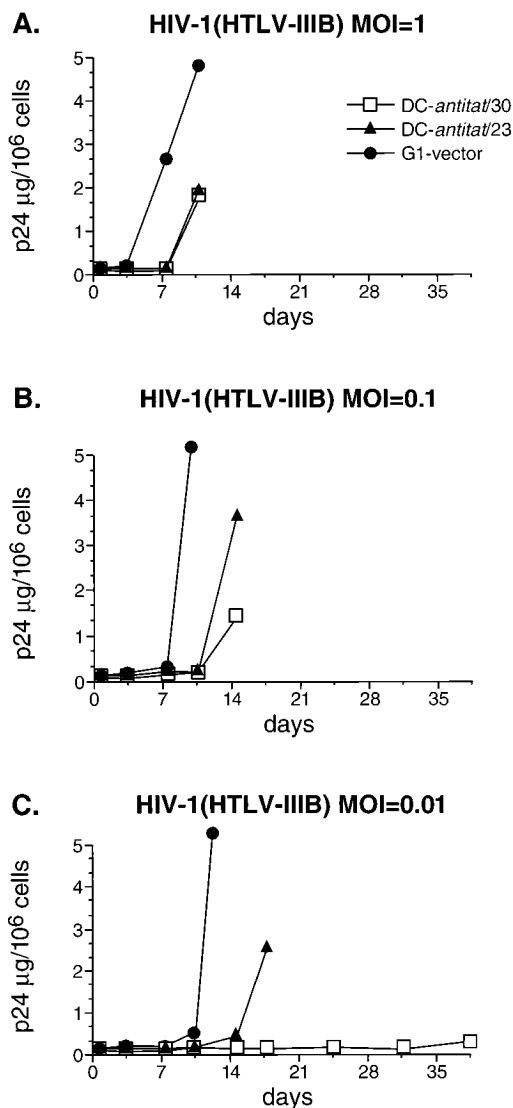


FIG. 3. Viral infectious dose-dependent inhibition of HIV-1 replication in *antitatt* gene-transduced Molt-3 cells. (A) Infection of *antitatt*-transduced cells with HIV-1_{HTLV-III_B} (MOI, 1). At 7 days, inhibition was increased after infection. DC-*antitatt*/30 and DC-*antitatt*/23 vectors blocked HIV-1 replication by 97 and 96%, respectively, compared with the G1 control vector. (B) Infection of *antitatt*-transduced cells with HIV-1_{HTLV-III_B} (MOI, 0.1). The greatest inhibition was detected 10 days after infection. The two *antitatt* vectors blocked HIV-1 replication by 99 and 98%, respectively, compared with the G1 control vector. (C) Infection of *antitatt*-transduced cells with HIV-1_{HTLV-III_B} (MOI, 0.01). The greatest inhibition was detected 14 days after infection. Cells transduced with DC-*antitatt*/30 did not produce detectable amounts of p24 until 31 days after infection. At day 14, DC-*antitatt*/23 vectors blocked HIV-1 replication by 97% compared with the G1 control vector.

after 200 days the inhibition exceeded 3,000-fold (p24 production by G1 vector-transduced cells was 419 ng/10⁶ cells versus 0.13 ng/10⁶ cells with the DC-*antitatt*/30). These results again suggest that the *antitatt* gene can be effective for long-term inhibition of HIV-1.

Retrovirus vector-mediated gene transfer into PBMCs. Retrovirus vector-mediated gene delivery is characterized by low-efficiency gene transfer into activated PBMCs. Therefore, to demonstrate the antiviral efficacy of the *antitatt* genes in these cells, augmentation of the relative amount of transduced cells is required. However, the presence of the neomycin phospho-

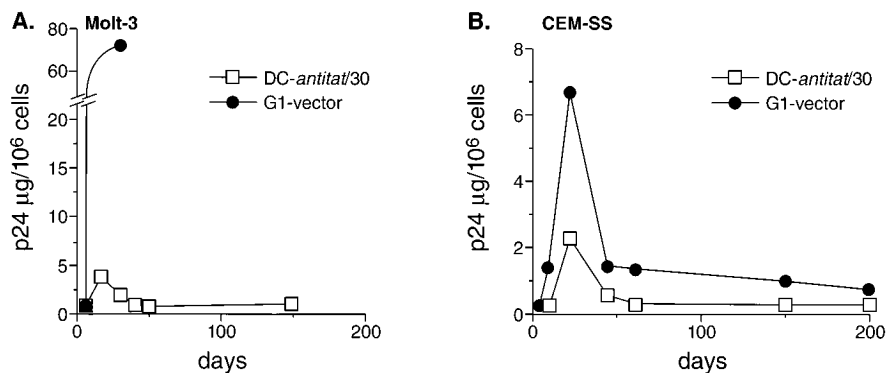


FIG. 4. Long-term antiviral effect of the *antitit* gene after high-MOI HIV-1 challenge. The *antitit* gene- and control vector-transduced cells were infected with HIV-1_{HTLV-III_B} (MOI, 1) and cultured for 5 months. HIV-1 replication was monitored by the p24 enzyme-linked immunosorbent assay (Coulter). (A) Long-term inhibition of HIV-1 replication in Molt-3 cells. (B) Long-term inhibition of HIV-1 replication in CEM-SS cells.

transferase gene in the retrovirus vectors permits cells to grow in medium supplemented with G418. Selection of primary lymphocytes is extremely difficult, because cells isolated from different donors have different sensitivities to G418 and because primary PBMCs can be grown with great difficulty with interleukin-2 for periods greater than a few months. PBMCs isolated from normal donors were transduced either with the *antitit* or with the control retrovirus-vector supernatants. The percentage of transduced PBMCs was estimated before and after selection by semiquantitative PCR with primers targeting the neomycin phosphotransferase gene. Figure 5 shows that under our experimental conditions, the efficiency of transduction of activated PBMCs by amphotropic retrovirus vectors was between 0.3 and 5% before selection, whereas G418 selection led to a significant increase (30%) of the transduced cell population.

Inhibition of HIV-1 replication in primary lymphocytes. Primary PBMCs are among the target cells of HIV-1 infection. To demonstrate the antiviral activity of *antitit* gene

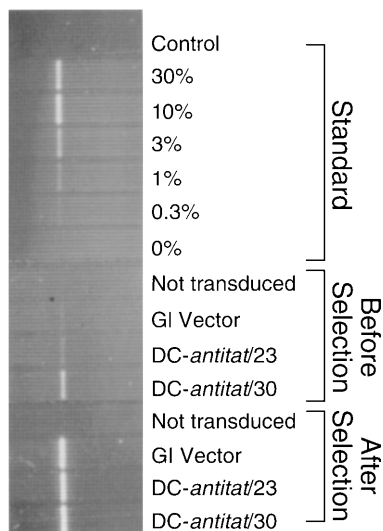


FIG. 5. Detection of the efficiency of retrovirus-mediated gene transfer into PBMCs and the enrichment of transduced cells after G418 selection. The percentage of transduced cells was determined by semiquantitative PCR. The transduction efficiency of activated PBMCs by amphotropic retrovirus vectors was between 0.3 and 5%, and G418 selection led to a significant increase (30%) in the transduced cell population.

therapy, retrovirus vector-transduced and G418-selected PBMCs were challenged with HIV-1_{HTLV-III_B}. *antitit* inhibited HIV-1_{HTLV-III_B} replication in primary cells (Fig. 6A) in a manner similar to the inhibition obtained with immortalized T-cell lines (Fig. 3C). The *antitit* gene oriented in the forward direction with respect to the vector backbone (DC-*antitit*/30) was a more effective inhibitor of HIV-1 replication in PBMCs than was the gene in the reverse orientation (DC-*antitit*/23). One possible reason for this difference is that DC-*antitit*/30 expression is not strictly dependent on Tat expression, because the full-length vector RNA transcribed from the 5' Moloney murine leukemia virus LTR also contains *antitit* sequences which should have antiviral activity. Presumably, the activity of DC-*antitit*/23 is dependent only on Tat expression. At lower HIV-1 challenge doses (MOI, 0.001), the antiviral activities of the two *antitit* vectors were the same, and both completely inhibited HIV-1 replication (p24 was not detectable 25 days after infection), in contrast to the control vector (281 pg of p24/10⁶ cells).

The *antitit* gene not only blocked HIV-1 replication but also protected CD4⁺ lymphocytes from the cytopathic effect of the virus. *antitit* and control vector-transduced primary lymphocytes were selected and infected with HIV-1_{HTLV-III_B}. At 3 weeks after challenge, CD4 and CD8 subsets were analyzed by flow cytometry. We found (Fig. 6B) that the *antitit* gene protected CD4⁺ cells, in contrast to the results with the control vector, when the majority of CD4⁺ cells were killed. These results demonstrate that *antitit* gene therapy not only inhibits HIV-1 replication and hinders virus spread but also prevents the killing of CD4⁺ lymphocytes.

Inhibition of the replication of a field isolate of HIV-1. One objection to the use of laboratory strains of HIV-1 for in vitro evaluation of antiviral agents is that passage through immortalized cell lines introduces the theoretical possibility of attenuation of the virulence of the HIV-1 isolate. Consequently, in addition to the above studies involving a common laboratory strain (HIV-1_{HTLV-III_B}), the antiviral activities of *antitit* vectors were evaluated by challenging the transduced cells with a primary clinical HIV-1 isolate (strain 3935; a gift from R. Redfield, Walter Reed Army Institute of Research, Rockville, Md., and P. Markham, Advanced BioSciences, Kensington, Md.). This HIV-1 isolate was never grown in immortalized cell lines but was passaged only in primary PBMCs. Normal PBMCs were transduced with *antitit* or control vectors, selected in G418 as above, and infected with HIV-1 3935. Both *antitit* vectors (forward and reverse orientations) inhibited

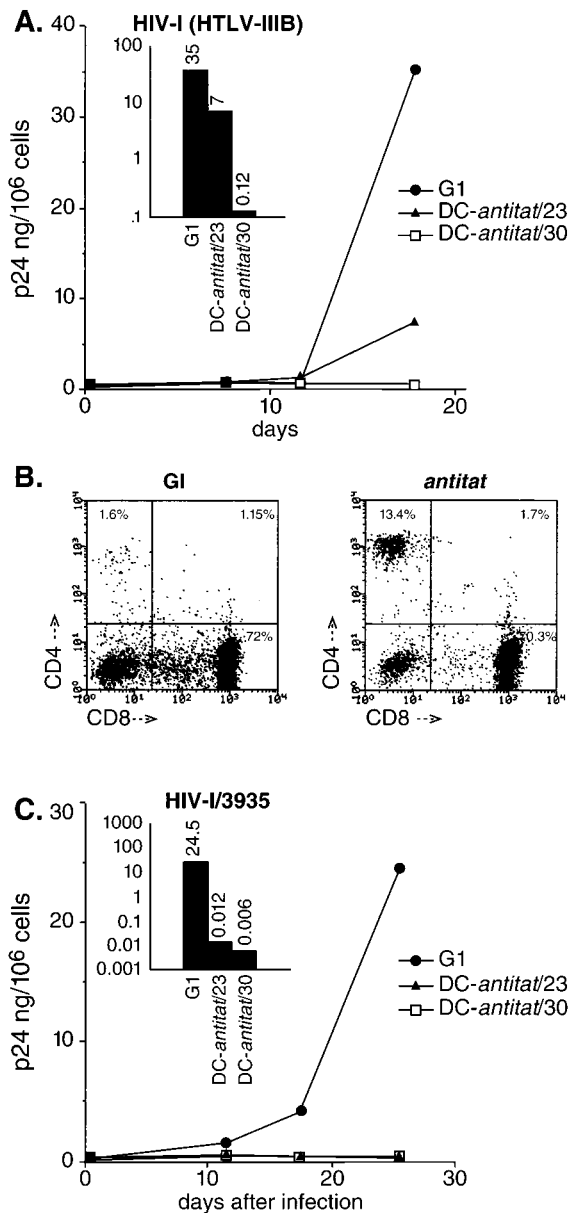


FIG. 6. Inhibition of HIV-1 replication by the *antitap* gene in PBMCs. (A) Inhibition of HIV-1_{HTLV-IIIb} replication in PBMCs. PBMCs transduced by *antitap* and control retrovirus vectors were selected with G418 and infected with HIV-1_{HTLV-IIIb}. The kinetics of virus replication in these cells are depicted. The insert shows the levels of p24 (in nanograms per 10⁶ cells) 17 days postinfection. (B) Protection of the CD4⁺ T-cell subset from the cytopathic effect of the virus. PBMCs were transduced by *antitap* (DC-*antitap*/23) and control (G1) retrovirus vectors, selected, and infected with HIV-1_{HTLV-IIIb}. At 3 weeks after virus challenge, subsets of CD4⁺ and CD8⁺ T cells were analyzed by flow cytometry. (C) Inhibition of the replication of a primary HIV-1 field isolate (HIV-1 3935). PBMCs were transduced with control and *antitap* retrovirus vectors. After G418 selection, cells were infected with a field isolate of HIV-1 3935. The kinetics of virus replication are depicted. The insert shows the levels of p24 (in nanograms per 10⁶ cells) 17 days postinfection.

HIV-1 3935 replication by more than 2,000-fold (Fig. 6C). These observations indicate that the *antitap* gene therapy is not limited to its effectiveness against a particular laboratory HIV-1 strain but that *antitap* is able to block replication of field isolates of HIV-1 obtained from infected individuals.

DISCUSSION

The *antitap* inhibitory gene has several important features relevant to its therapeutic potential. First, its expression is dependent on activation by Tat, and therefore the *antitap* gene should be expressed only in cells in which the Tat protein is present. We have previously demonstrated that polymeric TAR transcription from the HIV-1 LTR is very low in the absence of Tat and is highly activated if Tat is present (22). However, *antitap* not only is regulated by Tat but also inhibits Tat. This autocrine regulation provides a mechanism for amplifying and/or prolonging the response to Tat and does not permit overproduction of the inhibitory RNA when it is unnecessary, i.e., in uninfected cells. Since TAR can also bind cellular factors (16, 23, 31, 32, 36), the possibility that long-term, constitutive overexpression of TAR RNA will sequester some essential proteins could be a matter of concern. If these specific cellular factors play an important role in cellular regulation, the autocrine regulation of the *antitap* gene may be important in avoiding overexpression and the theoretical possibility of toxicity. Second, *anti-tat* is a dual-function gene, inhibiting HIV-1 Tat function through two different mechanisms, and the polymeric TAR and the AS-TAT on the same transcript have an additive inhibitory effect. Since Tat activates HIV-1 gene expression and the production of inflammatory cytokines (7, 8), reduction of Tat production results not only in inhibition of HIV-1 replication but also in inhibition of cytokine activation. Third, the low likelihood of simultaneous mutations of Tat and TAR during virus replication and the conserved structural requirements for their interaction should make viral escape less likely than with some other approaches to therapy. In support of this hypothesis, we have demonstrated that polymeric TAR is a general inhibitor of primate lentiretroviruses, inhibiting not only HIV-1 but also the simian immunodeficiency virus SIV_{Mac251} (21), and in the present work we have extended this result by showing inhibition of a field isolate of HIV-1. Fourth, polymeric-TAR binding to Tat in HIV-1-infected cells prevents its extracellular release and inhibits the activities of extracellular Tat taken up by cells (4, 12, 13). In this respect, we have previously demonstrated that polymeric TAR inhibits the activation of Tat-defective proviruses (9) and associated cellular alterations, both of which are induced by exogenous Tat (4, 12). Fifth, inhibition of Tat results in activation of negative-strand RNA transcripts from the 3' HIV-1 LTR (25). It is possible that this RNA acts as an antisense inhibitor, in addition to the AS-TAT provided in the *antitap* gene. A negative-strand RNA that reflects the perfect complementary sequence of any HIV-1 variants in the cell would, of course, have an advantage over the genetically engineered AS-TAT gene.

The antiviral effect of the *antitap* gene is not comparable to the inhibition of Tat transactivation induced by benzodiazepine compounds (Ro5-3335 and Ro24-7429) (19). These chemicals block Tat activation of transcription from the HIV-1 LTR, leading to inhibition of virus replication. However, there is increasing evidence that it is the interaction of Ro24-7429 with cellular factors rather than with Tat which is responsible for the antiviral activity of this compound (6, 18, 35). This may explain the toxicity of the benzodiazepine compounds and the discrepancies in results of efficacy studies.

Since the results with the Molt-3 cell line were similar to results with the primary PBMCs, the Molt-3 cells may be appropriate for initial preclinical biologic efficacy evaluations of HIV-1-inhibitory gene transfer strategies. The complete inhibition of HIV-1 replication in primary cells also confirms that the antiviral activity of the *antitap* gene is more efficient at

lower viral challenge doses and suggests that the spread of HIV-1 might be blocked *in vivo* by *antitAT* when the viral burden is low. Therefore, we propose that *antitAT* gene therapy will be more efficient in early intervention when the viral load is smaller (2, 11, 29).

There are different ways to use *ex vivo* gene therapy against HIV-1 infection. The simplest is to transduce the inhibitory gene into PBMCs or purified CD4⁺ lymphocytes isolated from HIV-1-infected individuals and to select and reinfuse these cells into the patient (3). The therapeutic efficacy of this approach will probably be very limited, mainly because of the low percentage of transduced T cells. Another approach is to introduce the inhibitory gene into hematopoietic stem cells. If stem cells will properly mature in patients with AIDS (5), genetically engineered cells could repopulate the immune system without allowing replication of the virus. This approach is attractive because, in theory, one treatment could lead to control of HIV-1; however, the present stem cell and gene transfer technologies have not yet been adequately developed to make this treatment feasible (34). The ultimate goal of stem cell therapy is successful repopulation with all hematopoietic cells containing the foreign gene. Since only a small fraction of these cells are permissive for HIV-1 replication, we think that it will be important to express the inhibitory gene only in cells which become infected. Regulation of the expression of the inhibitory gene may reduce potential toxicity problems, because an inactive gene should not interfere with the proper function of the cell. Therefore, we propose the autoregulated *antitAT* gene as a candidate for anti-HIV-1 gene therapy with lymphocytes now and with stem cells in the future as the technology is further developed.

ACKNOWLEDGMENTS

We thank E. Wolonowicz for technical help; B. White and R. Lyons for PCR and transduction experiments; P. Lusso and R. Crowley for FACS analysis; F. Lori, S. Arya, and P. Markham for helpful discussion; and L. Anderson for editorial help.

REFERENCES

- Arya, S. K., C. Guo, S. F. Josephs, and F. Wong-Staal. 1985. Transactivator gene of human T-lymphotropic virus type III (HTLV-III). *Science* **229**:69-73.
- Bagnarelli, P., S. Menzo, A. Valenza, A. Manzin, M. Giacca, F. Ancarani, G. Scalise, P. E. Varaldo, and M. Clementi. 1992. Molecular profile of human immunodeficiency virus type 1 infection in symptomless patients and in patients with AIDS. *J. Virol.* **66**:7328-7335.
- Baltimore, D. 1988. Gene therapy. Intracellular immunization. *Nature (London)* **335**:395-396.
- Barillari, G., L. Buonaguro, V. Fiorelli, J. Hoffman, F. Michaels, R. C. Gallo, and B. Ensoli. 1992. Effects of cytokines from activated immune cells on vascular cell growth and HIV-1 gene expression. Implications for AIDS-Kaposi's sarcoma pathogenesis. *J. Immunol.* **149**:3727-3734.
- Bonyhadi, M. L., L. Rabin, S. Salimi, D. A. Brown, J. Kosek, J. M. McCune, and H. Kaneshima. 1993. HIV induces thymus depletion *in vivo*. *Nature (London)* **363**:728-732.
- Braddock, M., P. Cannon, M. Muckenthaler, A. J. Kingsman, and S. M. Kingsman. 1994. Inhibition of human immunodeficiency virus type 1 Tat-dependent activation of translation in *Xenopus* oocytes by the benzodiazepine Ro24-7429 requires *trans*-activation response element loop sequences. *J. Virol.* **68**:25-33.
- Buonaguro, L., G. Barillari, H. K. Chang, C. A. Bohan, V. Kao, R. Morgan, R. C. Gallo, and B. Ensoli. 1992. Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines. *J. Virol.* **66**:7159-7167.
- Buonaguro, L., F. M. Buonaguro, G. Giraldo, and B. Ensoli. 1994. The human immunodeficiency virus type 1 Tat protein transactivates tumor necrosis factor beta gene expression through a TAR-like structure. *J. Virol.* **68**:2677-2682.
- Chang, H. K., R. Gendelman, J. Lisiewicz, R. C. Gallo, and B. Ensoli. 1994. Block of HIV-1 infection by a combination of antisense tat RNA and TAR decoys: a strategy for control of HIV-1. *Gene Ther.* **1**:208-216.
- Dayton, A. L., J. G. Sodroski, C. A. Rosen, W. C. Goh, and W. A. Haseltine. 1986. The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell* **44**:941-947.
- Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature (London)* **362**:359-362.
- Ensoli, B., G. Barillari, S. Z. Salahuddin, R. C. Gallo, and F. Wong-Staal. 1990. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature (London)* **344**:84-86.
- Ensoli, B., L. Buonaguro, G. Barillari, V. Fiorelli, R. Gendelman, R. A. Morgan, P. Wingfield, and R. C. Gallo. 1993. Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J. Virol.* **67**:277-287.
- Ensoli, B., R. Gendelman, P. Markham, V. Fiorelli, S. Colombini, M. Raffeld, A. Cafaro, H.-K. Chang, J. N. Brady, and R. C. Gallo. 1994. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. *Nature (London)* **371**:674-680.
- Feinberg, M. B., D. Baltimore, and A. D. Frankel. 1991. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc. Natl. Acad. Sci. USA* **88**:4045-4049.
- Frankel, A. D., and C. O. Pabo. 1988. Cellular uptake of the Tat protein from human immunodeficiency virus. *Cell* **55**:1189-1193.
- Gunnery, S., S. R. Green, and M. B. Mathews. 1992. Tat-responsive region RNA of human immunodeficiency virus type 1 stimulates protein synthesis *in vivo* and *in vitro*: relationship between structure and function. *Proc. Natl. Acad. Sci. USA* **89**:11556-11561.
- Hantzopoulos, P. A., B. A. Sullenger, G. Ungers, and E. Gilboa. 1989. Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. *Proc. Natl. Acad. Sci. USA* **86**:3519-3523.
- Hsu, M. C., U. Dhirga, J. V. Earley, M. Holly, D. Keith, C. M. Nalin, A. R. Richou, A. D. Schutt, S. Y. Tam, M. J. Pothash, D. J. Volsky, and D. D. Richman. 1993. Inhibition of type 1 human immunodeficiency virus replication by a tat antagonist to which the virus remains sensitive after prolonged exposure *in vitro*. *Proc. Natl. Acad. Sci. USA* **90**:6395-6399.
- Hsu, M. C., A. D. Schutt, M. Holly, L. W. Slice, M. I. Sherman, D. D. Richman, M. J. Potash, and D. J. Volsky. 1991. Inhibition of HIV replication in acute and chronic infections *in vitro* by a Tat antagonist. *Science* **254**:1799-1802.
- Lisiewicz, J., J. Rappaport, and R. Dhar. 1991. Tat regulated production of multimerized TAR RNA inhibits HIV-1 gene expression. *New Biol.* **3**:82-89.
- Lisiewicz, J., D. Sun, J. Smythe, P. Lusso, F. Lori, A. Louie, P. Markham, J. Rossi, M. Reitz, and R. C. Gallo. 1993. Inhibition of human immunodeficiency virus type 1 replication by regulated expression of a polymeric Tat activation response RNA decoy as a strategy for gene therapy in AIDS. *Proc. Natl. Acad. Sci. USA* **90**:8000-8004.
- Lori, F., J. Lisiewicz, J. Smythe, A. Cara, T. A. Bunnag, D. Curiel, and R. C. Gallo. 1994. Rapid protection against human immunodeficiency virus type 1 (HIV-1) replication mediated by high efficiency non-retroviral delivery of genes interfering with HIV-1 tat and gag. *Gene Ther.* **1**:27-31.
- Marciniak, R. A., M. A. Garcia-Blanco, and P. A. Sharp. 1990. Identification and characterization of a HeLa nuclear protein that specifically binds to the trans-activation response (TAR) element of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **87**:3624-3628.
- McLachlin, J. R., N. Mittereder, M. B. Daucher, M. Kadan, and M. A. Eglitis. 1993. Factors affecting retroviral vector function and structural integrity. *Virology* **195**:1-5.
- Michael, N. L., M. T. Vahey, L. D'Arcy, P. K. Ehrenberg, J. D. Mosca, J. Rappaport, and R. R. Redfield. 1994. Negative-strand RNA transcripts are produced in human immunodeficiency virus type 1-infected cells and patients by a novel promoter downregulated by Tat. *J. Virol.* **68**:979-987.
- Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* **6**:2895-2902.
- Morgan, R. A., and W. F. Adamson. 1993. Human gene therapy. *Annu. Rev. Biochem.* **62**:191-217.
- Neumann, J. R., C. A. Morency, and K. O. Russian. 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *BioTechniques* **5**:444-448.
- Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature (London)* **362**:355-358.
- Pauwels, R., J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, and E. DeClerq. 1988. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods* **20**:309-321.
- Rounseville, M. P., and A. Kumar. 1992. Binding of a host cell nuclear protein to the stem region of human immunodeficiency virus type 1 *trans*-activation-responsive RNA. *J. Virol.* **66**:1688-1694.
- Sheline, C. T., L. H. Milocco, and K. A. Jones. 1991. Two distinct nuclear transcription factors recognize loop and bulge residues of the HIV-1 TAR

- RNA hairpin. *Genes Dev.* **5**:2508–2520.
33. **Sodroski, J., R. Patarca, C. Rosen, F. Wong-Staal, and W. A. Haseltine.** 1985. Location of the trans-activating region of the genome of human T-cell lymphotropic virus type III. *Science* **229**:74–77.
 34. **Spain, L. M., and R. C. Mulligan.** 1992. Purification and characterization of retrovirally transduced hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **89**:3790–3794.
 35. **Witvrouw, M., R. Pauwels, A.-M. Vandamme, D. Schols, D. Reymen, N. Yamamoto, J. Desmyter, and E. De Clercq.** 1992. Cell type-specific anti-human immunodeficiency virus type 1 activity of the transactivation inhibitor Ro5-3335. *Antimicrob. Agents Chemother.* **36**:2628–2633.
 36. **Wu, F., J. Garcia, D. Sigman, and R. Gaynor.** 1991. Tat regulates binding of the human immunodeficiency virus trans-activating region RNA loop-binding protein TRP-185. *Genes Dev.* **5**:2128–2140.