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Additive and antagonist effects of therapeutic gene combinations for suppression of HIV-1 infection

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Abstract

A previously described Moloney-based vector expressing a double copy anti-*tat* antisense tRNA (DC-tRNA-AT) (Biasolo et al., 1996. J. Virol. 70, 2154–2161) was modified to increase the copy number of the antisense molecule and to target the intra-cytoplasmic localization of the HIV genome. To this end, an anti-U5 hammerhead ribozyme, engineered as a hybrid small adenoviral VAI RNA (VAIα), was inserted into the vector as a single molecule or in combination with the double copy anti-*tat* sequence. The retroviral vector expressing only VAIα (DC-VAIα) inhibited HIV-1 replication to an extent comparable to that of DC-tRNA-AT. A more effective inhibition was produced by the vector expressing multiple copies of the anti-*tat* antisense (DC-6tRNA-AT). This higher effectiveness correlated with anti-*tat* stochiometry, i.e. with the absolute number of therapeutic molecules being produced on a per cell basis at the steady state. Surprisingly, when the tRNA-AT and VAIα genes were combined in the same vector (DC-AT-VAIα), an enhancement of viral replication was noticed. This study indicates that it is possible to potentiate the antiviral activity of a retroviral vector by increasing the steady-state level of the therapeutic molecule. Results also show that the combined expression of two singularly active therapeutic RNAs can have antagonistic rather than synergistic effects.

Keywords: HIV-1 infection; Poly-gene therapy; Adoptive cell therapy; Antisense tRNA; VAI ribozyme

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1. Introduction

The use of highly active antiretroviral therapy (HAART) can reduce plasma HIV load to undetectable levels and rescue $CD4^+$ cell counts with significant benefit on overall morbidity and mortality in HIV seropositive patients. Recent studies have demonstrated that HIV establishes a latent infection in resting memory $CD4^+$ T-cells (Finzi et al., 1999; Zhang et al., 1999; Ramratnam et al., 2000). The persistence of these reservoirs has been observed in chronically infected individuals, irrespectively of whether they were anti-retroviral therapy naive or treated by HAART. It has been calculated that eradication of HIV infection by HAART could take as long as 60 years (Ho, 1998; Zhang et al., 1999; Ramratnam et al., 2000). Selection of viral resistant strains and occurrence of side-effects make such a long-term use of HAART problematic.

A number of gene therapy approaches to inhibit different steps of viral replication had already begun in the pre-HAART era in an attempt to combat AIDS. Effector gene products included antisense (Veres et al., 1996, 1998) and catalytic RNAs (ribozyme) (Lo et al., 1992; Rossi et al., 1992; Wang et al., 1998; Zhang et al., 1998), RNA decoys (Bohjanem et al., 1997; Gervaix et al., 1997), peptides with transdominant negative effect (Chinen et al., 1997; Stauber et al., 1998), intrabodies (Levin et al., 1997; Kitamura et al., 1999) and intrakines (Yang et al., 1997; Bai et al., 1998). With respect to proteins or peptides, RNA therapeutics have the advantage of being non-immunogenic and unable to elicit a harmful immune response that may have deleterious effects in vivo.

Previously, we showed the anti-HIV potency of a retroviral vector expressing a chimeric anti-*tat* antisense tRNA in double copy (DC-tRNA-AT) (Biasolo et al., 1996). The antisense sequence was directed against the first codon of HIV-1 *tat*. Among relevant HIV-1 transcripts or *cis*-acting structural and regulatory genomic sequences, *tat* is a key target for gene therapy not only because it is an early transactivator of the expression of all HIV-1 genes, but also because it may play an important role in the pathogenesis of AIDS and associated malignancies (Dayton et al., 1986). The tRNA, as a carrier molecule, conferred to the antisense high level of expression, stability, active conformation and subcellular localization with the target substrate. We were actually the first to propose the use of gene therapy of AIDS in the form of an adoptive immunotherapy, as an adjuvant to chemotherapy (Manca et al., 1997). For this approach, we used HIV-genetically resistant $CD4^+$ cell lines, specific for antigens of HIV-1 and opportunistic pathogens. These lines, obtained from normal donors and HIV-infected individuals, were not only refractory to HIV replication but retained their biological and functional properties after transduction (Manca et al., 1997, 1999).

An effective gene therapy of HIV infection requires high expression levels of the therapeutic transgenes and targeting major steps of the virus infectious cycle. This is especially true for antigen-specific $CD4^+$ cells adopted for in vivo transfer since these cells need to be highly resistant to HIV-1 infection after infusion in a seropositive patient. As an extension of our previous experiments, in this study we attempted to improve the antiviral activity of the DC-tRNA-AT by introducing six copies of the anti-*tat* tRNA into the vector and associating this antisense with a ribozyme directed against a highly conserved sequence on the U5 region of the viral genome. The combination of genes directed against different viral targets may improve gene therapy of AIDS not only by creating synergistic inhibitory effect, but also by decreasing the probability of generating escape mutants. Furthermore, ribozymes can target the HIV replication cycle both in the pre-integration and post-integration phase by cleaving incoming RNA as well as transcribed genomic and subgenomic mRNAs. Recombinant retroviral vectors, containing: (i) two and 12 copies of the anti-*tat* gene; (ii) two copies of the anti-*tat* and one copy of the anti-U5 gene with different orientations and (iii) one copy of the anti-U5 gene alone, were constructed. The anti-HIV-1 efficacy of these constructs has been tested in Jurkat cell lines. The expression of the therapeutic genes has also been analyzed.

2. Materials and methods

2.1. Construction of retroviral vectors

The main features of the vectors, in the final form of the proviral DNA present in target cells after retrotranscription, are shown in Fig. 1. Generation of the tRNA-AT, a $tRNA^{pro}$ having the anticodon loop replaced by a 20-nucleotide *tat* antisense sequence, and construction of the vector DC-tRNA-AT have been previously described (Biasolo et al., 1996). To obtain the DC-6tRNA-AT vector, the 96-bp DNA fragment coding for the anti-*tat* tRNA was subcloned into pSP73 vector (Promega), 3′ to the SP6 promoter, to yield plasmid pSP73-AT. The tRNA-AT sequence was excised and cloned into pSP73-AT to obtain a plasmid containing multiple copies of the therapeutic gene. The sequence coding for the six tRNA-AT was then cloned as an EcoRI–BamHI fragment into the BglII–SnaBI polylinker site of the 3′ long terminal repeat of the DCN2A retroviral vector (kind gift of E. Gilboa, Duke University), to yield plasmid DC-6tRNA-AT.

The sequence coding for the VAIα hammerhead (Prislei et al., 1997) was subcloned into plasmid pSP73-AT. The same VAIα sequence was then cloned as a SalI–XhoI fragment into the XhoI site of the DC-tRNA-AT vector at the 3′ end of the *neo* gene. Two constructs were obtained: DC-AT-VAIα (5′–3′) and DC-AT-VAIα (3′–5′) which differed for the orientation of the VAIα gene with respect to the LTR promoter of the vector. With the same strategy the VAIα sequence was cloned into the DCN2A retroviral vector, to obtain the DC-VAIα construct.

2.2. Preparation of cell lines expressing the therapeutic genes

PA317 packaging cells were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml at 37 °C with 5% CO_2.

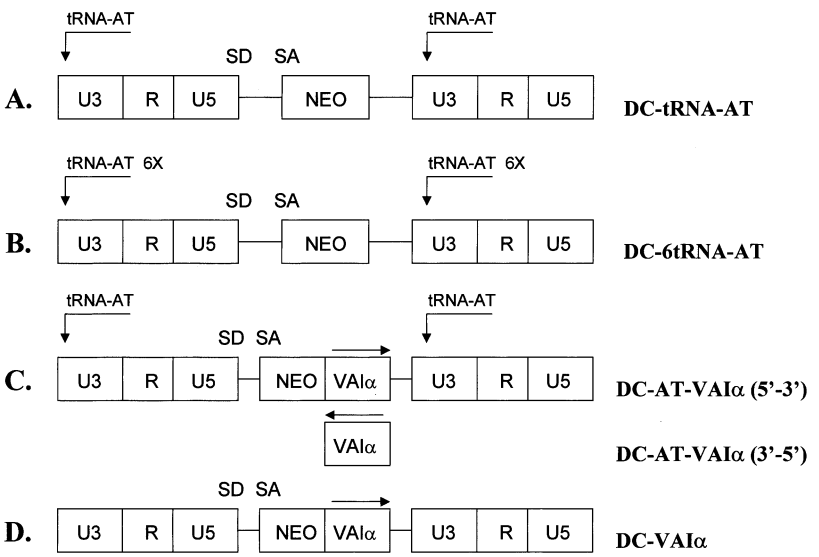


Fig. 1. Schematic representation of retroviral vectors. pDCN2A is the parental MoMLV-based retroviral vector. (A) DC-tRNA-AT: vector containing the chimeric antisense tRNA in the U3 region of the 3′ LTR. Since the U3 region of the 3′ LTR of retroviruses is duplicated at the 5′ LTR during the step of retrotranscription, the tRNA cassette is also present at the 5′ LTR after transduction of the target cells following retrotranscription. (B) DC-6tRNA-AT: vector containing six copies of the chimeric tRNA. All the copies of the therapeutic gene were inserted in the U3 region of the 3′ LTR of the vector. The proviral DNA is shown. (C) DC-AT-VAIα (5′–3′) and DC-AT-VAIα (3′–5′): vectors containing the anti-*tat* tRNA in combination with VAIα, an hammerhead ribozyme directed against the U5 region of HIV-1. These two vectors differ for the orientation of the VAIα with respect to the 5′ LTR promoter. (D) DC-VAIα: vector expressing VAIα.

Thirty-μg aliquots of DC-6tRNA-AT, DC-tRNA-AT, DC-AT-VAIα (5′–3′), DC-AT-VAIα (3′–5′) and DC-VAIα vectors were transfected into PA317 packaging cells by a calcium phosphate transfection system (Bethesda Research Laboratories) as instructed by the manufacturer. PA317 cells that stably produced amphotropically packaged DC-6tRNA-AT, DC-tRNA-AT, DC-AT-VAIα (5′–3′), DC-AT-VAIα (3′–5′) and DC-VAIα vectors were obtained by selection with 1 mg/ml of G418 (Sigma). For transduction, Jurkat cells cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (as described above), were incubated with 0.45-μm-pore-size-filtered supernatant from confluent vector-producing PA317 cells for 18 h in the presence of 4 μg/ml of Polybrene. Cells were then selected with 1 mg/ml of G418 (Sigma). Selection medium was changed every 3–4 days.

2.3. HIV-1 infection

HIV-1 virus stock was produced by transfection of Jurkat cells with the pSVC21 plasmid containing the infectious HXB2 molecular clone of HIV-1 (Ratner et al., 1985). Viral titre was measured as $TCID_{50}$/ml on C8166 cells using the Reed and Muench method (Jawetz et al., 1980). G418-selected transduced Jurkat cells and control cells (untransduced Jurkat cells) were incubated with the infectious strain at multiplicities of infection (m.o.i.) of 0.001 and 0.01 for 16 h and then washed twice. Generally every 3 days post-infection (p.i.), the infected cells were split with fresh medium to maintain a constant cell density. The culture supernatants were collected at different days p.i., filtered on 0.45-μm-diameter Millipore filters, and the reverse transcriptase activity was measured as previously described (Rho et al., 1981).

Syncytium formation was monitored in cell culture by visual inspection using a Leica DMIL inverted microscope at 3, 7, 14, 21 and 55 days after infection. The number of syncytia was scored and normalized to that observed for the untransduced Jurkat cells.

2.4. RNA reverse transcription and PCR amplification

Expression of VAIα and/or tRNA-AT by transduced cells (J-DC-AT-VAIα (5′–3′), J-DC-AT-VAIα (3′–5′), J-DC-VAIα and J-DC-tRNA-AT) was detected by PCR combined with reverse transcription (RT). Total RNA was extracted from Jurkat cells by the RNAzol B method (TEL-TEST). After treatment with RNase-free DNase (Boehringer), 1 μg of total RNA was PCR amplified with RT, using the forward primer VAIα 5′-TCTTCCGTGGTCTGGTGGAT-3′ and the reverse primer VAIα 5′-CACACCTGGGTAC-CGTGTGC-3′ for VAIα; the forward primer tRNA-AT 5′-GGCCGAATGGTCTAGTGG-3′ and the reverse primer tRNA-AT 5′-TTTCATT-GCCAAGTTTGTTT-3′ for tRNA-AT (Biasolo et al., 1996). PCR was carried out according to manufacturer's instructions (Gene Amp PCR reagent kit/Perkin Elmer). Fifteen μl of each PCR product was subjected to 3% gel electrophoresis and visualized under a UV lamp after staining with ethidium bromide. The amplified fragments were of 156 and 51 bp, for VAIα and tRNA-AT, respectively.

2.5. Real-Time PCR

The expression level of the anti-*tat* antisense RNA was evaluated with a real-time PCR assay. Briefly, total RNA was extracted from 5×10^7 Jurkat cells expressing two copies of the tRNA-antisense (J-DC-tRNA-AT) and from 5×10^7 Jurkat cells expressing 12 copies of the tRNA-antisense (J-DC-6tRNA-AT) by the RNAzol B method (TEL-TEST). After treatment with RNase-free DNase (Boehringer), 5 μl of total RNA was PCR amplified, after retrotranscription, using random primers (PE Applied Biosystem). In order to normalize the RNA amount of the extracted samples, a real-time PCR quantification of the human β-actin cDNA was performed with Taqman β-actin reagents, according to manufacturer's instructions (PE Applied Biosystem).

The real-time PCR (Rt-PCR) reactions were

performed in 100 μl final volume utilizing cDNA from J-DC-6tRNA-AT and J-DC-tRNA-AT, the forward primer tRNA-ATM 5′-GGCCGAATGGTCTAGTGGTA-3′ and the reverse primer tRNA 20 5′-CCTTTCATGCCAAGTTTGCT-3′, 0.6 mM (each) of dATP, dCTP, dGTP and dUTP, 100 mM KCl, 10 mM Tris–HCl, 20 mM EDTA, 10 mM $MgCl_2$, 2 U of *Taq* polymerase and the SYBR-green reagents according to manufacturer's instructions (PE Applied Biosystem). The amounts of cDNA used in this assay were chosen in order to maintain the threshold cycle (C_T) value in the linear range. Undiluted and diluted samples from J-DC-tRNA-AT and J-DC-6tRNA-AT were analyzed in duplicate. PCR mixtures were incubated at 95 °C for 5 min and then cycled at 95 °C for 10 s and at 60 °C for 45 s for a total of 40 cycles in an ABI PRISM 7700 Sequence Detection system. The anti-*tat* antisense amount was evaluated by interpolation over a standard curve made with the DC-tRNA-AT plasmid.

2.6. Heteroduplex tracking assay (HTA)

PCR products were analyzed by HTA using a radiolabeled ssDNA probe as described (Radaelli et al., 1998). Briefly, a dsDNA was amplified using plasmid pSP73tRNA-AT with the forward V63 ^{32}P-labeled primer and the reverse V64 biotinylated primer. Amplification was carried out in a final volume of 50 μl, in a mixture containing 1 μM of each primer, 200 μM of dATP, dCTP, dGTP and dUTP, 1.25 U of Taq DNA polymerase (Perkin Elmer), 2.25 mM $MgCl_2$, 50 mM KCl and 10 mM Tris–HCl (pH 8.3). PCR mixtures were incubated at 94 °C for 30 s, at 62 °C for 30 s and at 72 °C for 20 s, for a total of 30 cycles. The 51 bp fragment was purified from agarose gel with Jetsorb (Genomed). The single-stranded probe was obtained by denaturation and removal of the biotinylated strand after incubation with M-280 streptavidin-bound magnetic beads (Dynabeads, Dynal) using a magnetic particle concentrator (MPC-1, Dynal). RNA extracted from J-DC-6tRNA-AT and J-DC-tRNA-AT was amplified by RT-PCR using the forward and the reverse tRNA-AT primers. Amplification products were annealed to the ^{32}P-labeled ssDNA probe. In particular, heteroduplexes were formed by denaturation and reannealing of 5 μl of each PCR amplified product in a 10 μl mixture containing annealing buffer (1 mM NaCl, 100 mM Tris–HCl pH 7.8, 20 mM EDTA) and 5000 cpm/sample ^{32}P-labeled ssDNA probe. Denaturation was performed at 95 °C for 2 min, followed by 5 min incubation at 4 °C in a thermocycler. Single-stranded DNA probe alone (5000 cpm) and 5 μl of homoduplex control sample derived from amplification of plasmid pSP73tRNA-AT using unlabeled primers, were denatured and cooled in the same manner. All samples were loaded onto a 6% neutral 1 mm-thick polyacrylamide gel (30:0.8% acrylamide/bisacrylamide) in TBE buffer (88 mM Tris-borate, 2mM EDTA, pH 8) and run in TBE buffer on a Protean II vertical gel apparatus (Biorad) for 50 min at 500 V in a cold room. After separation, the heteroduplexes were visualized by autoradiography. In order to compare heteroduplexes' signal intensities, quantitative densitometric analysis was performed by using NIH Image J 1.23 image analysis program, which allows optical density readings conversion from pixel values.

2.7. Statistical analysis of antiviral activity

To investigate mathematically the antiviral effect of the therapeutic molecules, the relationship between viral growth expressed as HIV-1 reverse transcriptase activity (RT, quantified as cpm × 10^3/ml) and time (days after infection) was investigated by non-linear analysis. The process was conveniently described by a symmetric sigmoid (logistic) curve:

$$y = \frac{a}{1 + \exp\left(-\frac{x-b}{c}\right)}$$

where y was the RT level, x was the independent variable (time), a was the transition height (max level of RT at plateau), b and c were time constants. In particular, b was the transition center and c was linked to the initial doubling time t_2 by the relation $t_2 = 0.693 \times c$. The curves reflecting the different experimental conditions were com-

pared to those of Jurkat cells (at two m.o.i. levels), taken as the control curves. Statistical comparisons, based on Student's *t* test (eight degrees of freedom), were conducted on transition centers. A significant positive difference between experimental and control values indicated inhibition of HIV-1 replication, whereas a significant negative difference was taken as a proof of an enhancing effect.

3. Results

3.1. Construction of retroviral vectors and generation of stably transduced Jurkat cells

To increase anti-HIV-1 activity of previously developed DC-N2A-based retroviral vectors in our laboratory (Biasolo et al., 1996), new derivatives were designed which expressed six copies of the original anti-*tat* antisense sequence, as RNA polymerase III independent transcription unit (Fig. 1). Moreover, the combination of the anti-*tat* antisense with a U5 hammerhead ribozyme was also investigated in order to target different steps of the HIV-1 replication cycle. To this end, a VAIα hammerhead ribozyme, directed against the U5 region of the HIV-1 genome, was inserted into the same vector in combination with the tRNA-AT (Fig. 1). The ribozyme cleaved HIV RNA at position +115/+116 relative to the transcription initiation site of the HIV-1 clone HXB2, a highly conserved, accessible region present in all HIV RNAs. To analyse the possible effects of vector design on antiviral efficiency, we obtained two double-gene constructs that differed by the orientation of the ribozyme with respect to the 5′LTR promoter of the vector. A retroviral vector expressing the VAIα alone was also constructed. In all these vectors the ribozyme was inserted at the 3′ end of the *neo* gene (Fig. 1).

DC-6tRNA-AT, DC-AT-VAIα (5′–3′), DC-AT-VAIα (3′–5′) and DC-VAIα vectors were packaged as amphotrophic viruses in PA317 cells and used to transduce Jurkat cells; J-DC-6tRNA-AT, J-DC-AT-VAIα (5′–3′), J-DC-AT-VAIα (3′–5′) and J-DC-VAIα polyclonal cultures were selected in the presence of G418.

3.2. Expression of the transgenes in transduced Jurkat cells, Heteroduplex tracking assay and Real-time PCR

It has been shown that rearrangements and deletion might occur in retrovirus-based double copy vector (Junker et al., 1995). In addition, the viral transcriptional sequences for the expression of a desired gene product might undergo down-regulation over time. To specifically examine expression of anti-*tat* antisense and VAIα genes, driven by the $tRNA^{pro}$ and VAI promoters, respectively, stably transduced Jurkat cells were analysed by RT-PCR using specific primers. The amplified product (expected size, 51 and 156 bp fragments, specific for the antisense sequence and for VAIα) were detected only when PCR was carried out after RT and as long as 3 months after transduction with the recombinant vectors (Fig. 2 and data not shown). Similar results were obtained with J-DC-AT-VAIα (5′–3′), J-DC-VAIα and J-DC-tRNA-AT (data not shown).

To further investigate the steady-state level of the anti-*tat* antisense present in cells transduced by the double (DC-tRNA-AT) and multiple (DC-6tRNA-AT) copy vector, a heteroduplex assay was performed. Quantitative analysis of HTA gel images indicated that the presence of multiple copies of the transgene in the vector can increase the expression level of the therapeutic molecule by at least 5-fold (optical density readings were 7711 and 1402 for the DC-6tRNA-AT and the DC-tRNA-AT vectors, respectively) (Fig. 3).

To confirm that a higher expression of the anti-*tat* antisense molecule was driven by the DC-6tRNA-AT vector compared to the DC-tRNA-AT vector, a quantitative real-time PCR (Rt-PCR) was also performed. The differences in the steady-state level of the therapeutic antisense RNA in cells transduced with DC-6tRNA-AT versus those transduced with DC-tRNA-AT was this time about 10-fold (Fig. 4). On an average per cell basis, about 600 and 6000 anti-*tat* antisense RNA molecules were produced by the DC-tRNA-AT and DC-6tRNA-AT vector, respectively. Since our Jurkat cells were not a clonal population, the real absolute amount of intracellularly produced molecules may vary from cell to cell.

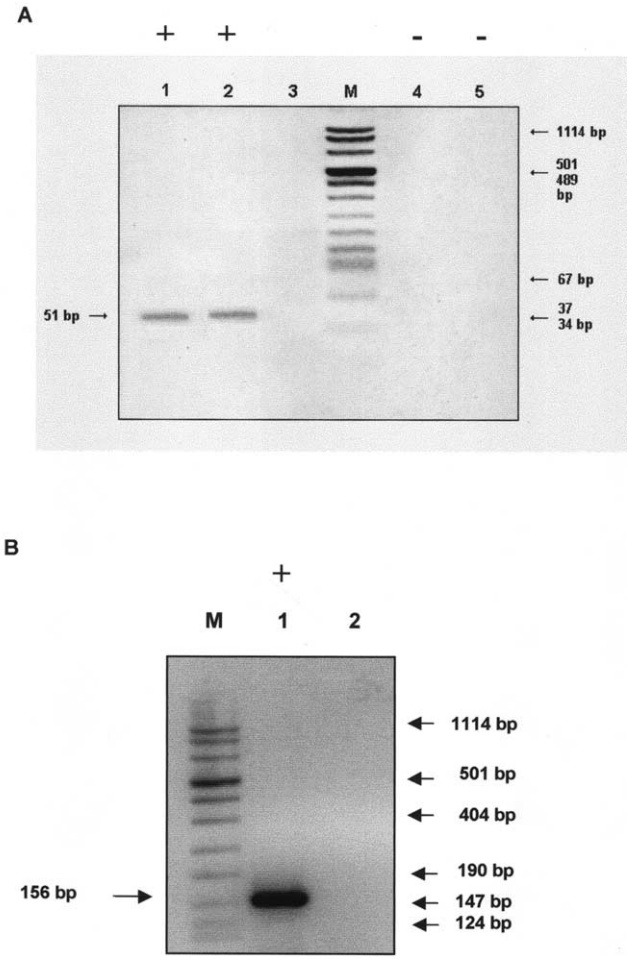


Fig. 2. Expression of anti-*tat* tRNA and VAIα in stable transduced J-DC-AT-VAIα (3′–5′). To investigate expression of both therapeutic RNAs, PCR analysis was performed combined with (+) (panel A, lanes 1 and 2; panel B, lane 1) or without (−) RT (panel A, lanes 4 and 5). For anti-*tat* tRNA the set of specific primers used was forward primer tRNA-AT and reverse primer tRNA-AT; for VAIα the set of primers was forward primer VAIα and reverse primer VAIα (refer to Section 2 for the exact sequence of these primers). Panel A, tRNA; panel B, VAIα. Panel A, lane 3, panel B, lane 2: control untransduced Jurkat cells. The size (in base pairs) of the amplified products is marked. M, size markers.

3.3. Inhibition of HIV-1 replication

Jurkat cells were transduced with the recombinant vectors and infected with HIV-1 at different m.o.i.s (0.001 and 0.01). To evaluate HIV-1 replication over time, RT activity in the cell culture supernatants and syncytium formation were monitored (see Section 2). The ability of these new constructs to inhibit HIV-1 replication was compared with that of the original DC-tRNA-AT vector. As depicted in Fig. 5, the presence of multiple copies of the anti-*tat* sequence conferred to transduced cells a higher resistance to HIV-1 replication that was inhibited for up to 17 days in Jurkat cells infected at a m.o.i of 0.01 (panel B); no RT activity was detectable for the length of the experiment (55 days) in cells infected at m.o.i of 0.001 (Fig. 5A and data not shown). Cytopathic effects (syncytium formation) appeared after 2 weeks from challenge in Jurkat cells transduced with the DC-tRNA-AT vector; no signs of cytopathicity were observed in Jurkat cells transduced

with the new DC-6tRNA-AT vector until day 55 of observation (data not shown). By converse, control cultures (non-transduced Jurkat cells) showed syncytia within 1 week following infection (not shown).

Jurkat cells transduced with the DC-VAIα vector, containing a VAIα hammerhead ribozyme directed against the U5 region of the HIV-1 genome, were similarly evaluated for their susceptibility to HIV-1 infection. RT activity was undetectable for about 15–17 days post-infection (m.o.i.s of 0.01 and of 0.001) (Fig. 5, panels C and D). Syncytia appeared in cell cultures at about 14 days after HIV-1 challenge at both m.o.i.s (data not shown).

Surprisingly, no inhibitory effect was seen when the two transgenes, tRNA-AT and anti-U5 VAI ribozyme, were combined; on the contrary, an increase of viral replication was noticed (Fig. 5, panels C and D). The effect was independent from the orientation of the ribozyme with respect to the LTR promoter of the vector. At an m.o.i. of 0.01, RT activity was detectable in the culture supernatants of transduced and non-transduced cells at 6 and 9 days after HIV-1 challenge, respectively; similar data were obtained when an m.o.i. of 0.001 was utilized. Results of the RT activity assay correlated with syncytium formation, since the cytopathic effects appeared 3 days post-infection and progressed more rapidly in Jurkat cells expressing the two effector molecules (data not shown).

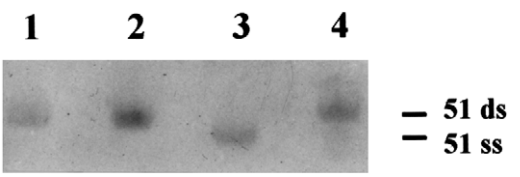


Fig. 3. Heteroduplex tracking assay. The duplexes formed between the ^{32}P-labeled ssDNA probe and the products of forward and reverse tRNA-AT primers obtained after RT-PCR of RNA extracted either from Jurkat cells transduced by the retroviral vectors DC-tRNA-AT (J-DC-tRNA-AT) and DC-6tRNA-AT (J-DC-6tRNA-AT) are shown. After hybridization, products were resolved on polyacrylamide gels and autoradiographed (see Section 2). Lane 1, J-DC-tRNA-AT; lane 2, J-DC-6tRNA-AT. The radioactive ssDNA probe was loaded on lane 3 and the positive control, obtained by hybridization of the ssDNA and the PCR amplification product from plasmid pSP73/tRNA-AT, on lane 4. A representative experiment is shown.

Our statistical analysis indicate that reverse transcriptase activity increased monotonically in function of time. All curves of replication, except one, showed a good agreement with the logistic model. Statistical comparisons to the Jurkat cells (control) at the m.o.i. levels investigated (0.01 and 0.001) are indicated in the Table 1. The DC-tRNA-AT, the DC-6tRNA-AT and the DC-VAIα vectors exerted an inhibitory effect on HIV-1 replication, whereas the DC-AT-VAIα (3′–5′) and the DC-AT-VAIα (5′–3′) vectors induced an enhancement. This reversal of effect demonstrates an antagonism between the two genetic components AT and VAIα when co-present in the same construct.

The DC-6tRNA-AT construct was more effective than the DC-tRNA-AT vector in inhibiting HIV-1 replication. This was demonstrated by a b-value of 21.7 versus 10.7 days at m.o.i. = 0.01 ($P = 0.045$). At m.o.i. = 0.001 the inhibition by the DC-6tRNA-AT was practically complete. When the DC-tRNA-AT vector was employed, the RT values achieved a plateau level, albeit slowly (b-value = 24.4 days).

4. Discussion

We recently proposed in the literature a new strategy of adoptive immunotherapy based on the ex vivo transfer of autologous CD4$^+$ T-cell lines, genetically resistant to HIV-1, which recognize specific antigens of HIV-1 and of opportunistic pathogens (Manca et al., 1997, 1999). The anti-HIV-1 gene was an anti-*tat* antisense expressed as a tRNA hybrid molecule by a Moloney-based vector. The antigen-specific CD4$^+$ lymphocytes, obtained from HIV-infected and non-infected individuals, were resistant to HIV-1 replication while maintaining their immunofunctional properties (Manca et al., 1997, 1999).

In the present work, we proposed to explore the combination of multiple anti-HIV-1 genes in order to increase the efficiency of our therapeutic

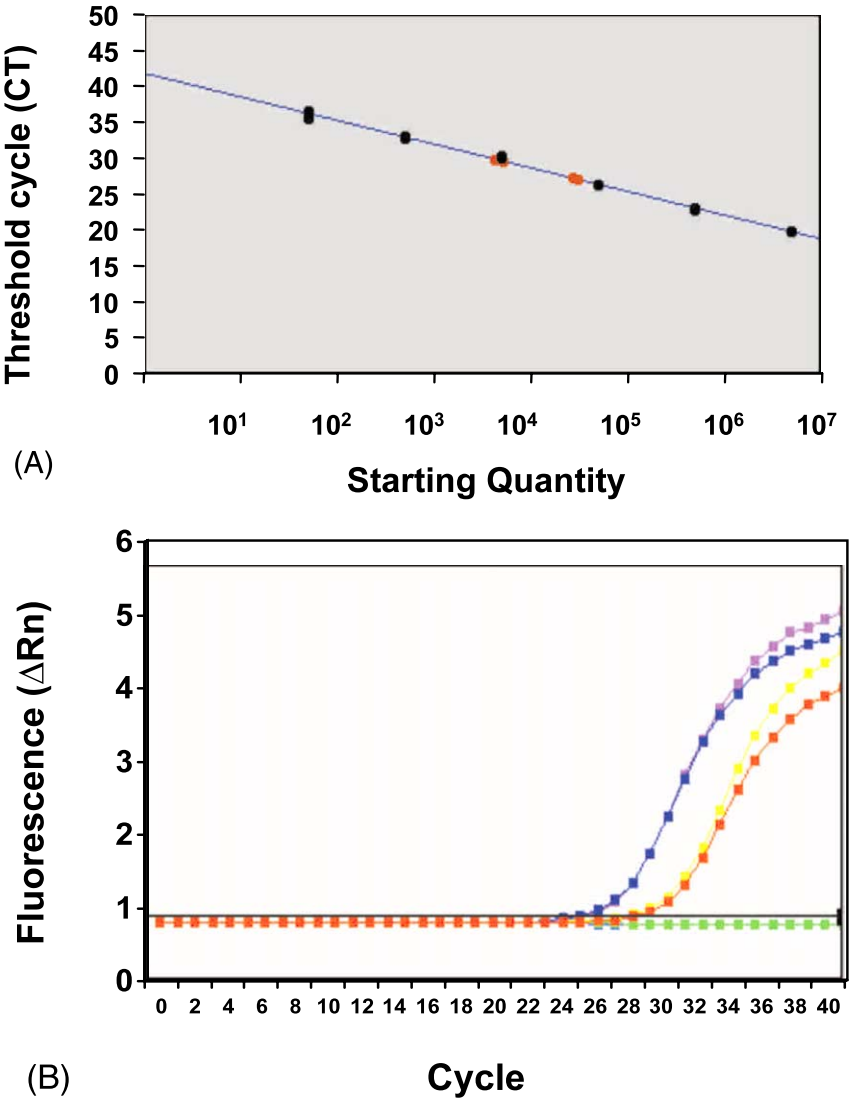
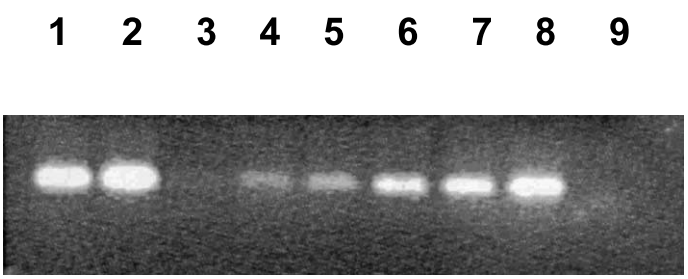


Fig. 4. Standard curve (panel A) obtained with the control plasmid DC-tRNA-AT and amplification plots (panel B) of real time PCR performed using cDNAs from J-DC-6tRNA-AT and J-DC-tRNA-AT. The expression of the multiple copy vector (purple and blue lines) is about 10-fold with respect to the double copy vector (yellow and orange lines) (see Section 2 for details). As a control of the specificity of the amplified product detected by SYBR-green staining, the real time PCR products were loaded on agarose gel (panel C).

approach. We were interested in obtaining a high level of therapeutic gene expression being conscious that this is a very important step toward a successful cell and gene therapy approach. Moreover, we wanted to target not only the efferent phase of the virus life cycle but, possibly, also the initial (afferent) phase so as to completely abort viral infection in the immune-armed effector cells.

The use of the human lymphoblastoid Jurkat cell line, which exhibits a high proliferative rate and is susceptible to transduction, allowed us to evaluate the efficacy of our strategy in a surrogate model of an antigen-specificT-cell.



(C)

Fig. 4. *continued*

Our results showed that Jurkat cells stably transduced with the vector containing 12 gene copies of the antisense tRNA (DC-6tRNA-AT) were more refractory to HIV-1 de novo infection than cells transduced with the original DC-tRNA-AT (containing 2 gene copies of the antisense tRNA). All inhibitory effects were m.o.i. dependent thus implying that a critical number of effector molecules needed to be intracellularly produced to antagonize HIV-1. By the use of two different RNA quantitation techniques, we examined whether a quantitative increase in the expression of the anti-*tat* antisense RNA could have influenced the antiviral activity in the transduced cells. Our data indeed confirmed that resistance to HIV-1 replication and cytopathicity correlated with the absolute concentration of the therapeutic RNA at the steady state level. A number from 600 (DC-tRNA-AT) to 6000 (DC-6tRNA-AT) molecules of anti-*tat* antisense RNA were produced on an average per cell basis, as assessed by real time PCR. This is the first time that a real quantitation of an inhibitory RNA molecule is obtained in cells undergoing genetic treatment. These results therefore validate the assumption that suppressive anti-HIV gene therapy based on antisense RNAs, to be efficacious, needs to yield intracellular numbers of effector molecules exceeding those of target molecules (Wang and Dolnick, 1993; Gervaix et al., 1997). The correlation we found between anti-HIV-1 activity and therapeutic gene expression confirm experiments of relative quantitation described by Gervaix et al. using RT-PCR and limiting dilutions (Gervaix et al., 1997). Since in our investigation different amounts of the same molecule (anti-*tat* antisense RNA) were expressed by the two retroviral vectors, the higher anti-HIV activity produced in cells transduced by DC-6tRNA-AT has to be ascribed to an additive inhibitory effect.

The intracellularly expressed anti-*tat* antisense RNA could not prevent the establishment of HIV-1 infection, as evidenced by the presence of proviral DNA in infected cells (not shown). In order to prevent proviral formation, we combined in the same vector the tRNA-anti-*tat* antisense with a hammerhead version (VAIα) of an hairpin ribozyme targeting the U5 region of the HIV-1 5′ long terminal repeat (LTR) in the cytosol (Prislei et al., 1997). For properly evaluating the effects of this combined gene therapy, retroviral vectors were designed to express the antisense and the ribozyme as individual effector RNA molecules,

transcribed under the control of the $tRNA^{pro}$ and VAIα promoters, respectively. Indeed, inhibition of HIV-1 replication and protection from cytopathic effects were observed when the VAIα hammerhead ribozyme was retrovirally expressed in transduced cells as the only therapeutic gene (Fig. 5). To our surprise, the dual function vector did not inhibit HIV-1 replication in transduced cells, but rather stimulated an increased level of viral growth. This result was not affected by the orientation of the ribozyme with respect to the LTR promoter, thus implying that transcription orientation and promoter use were not likely to play a role in the observed phenomenon. A clear an

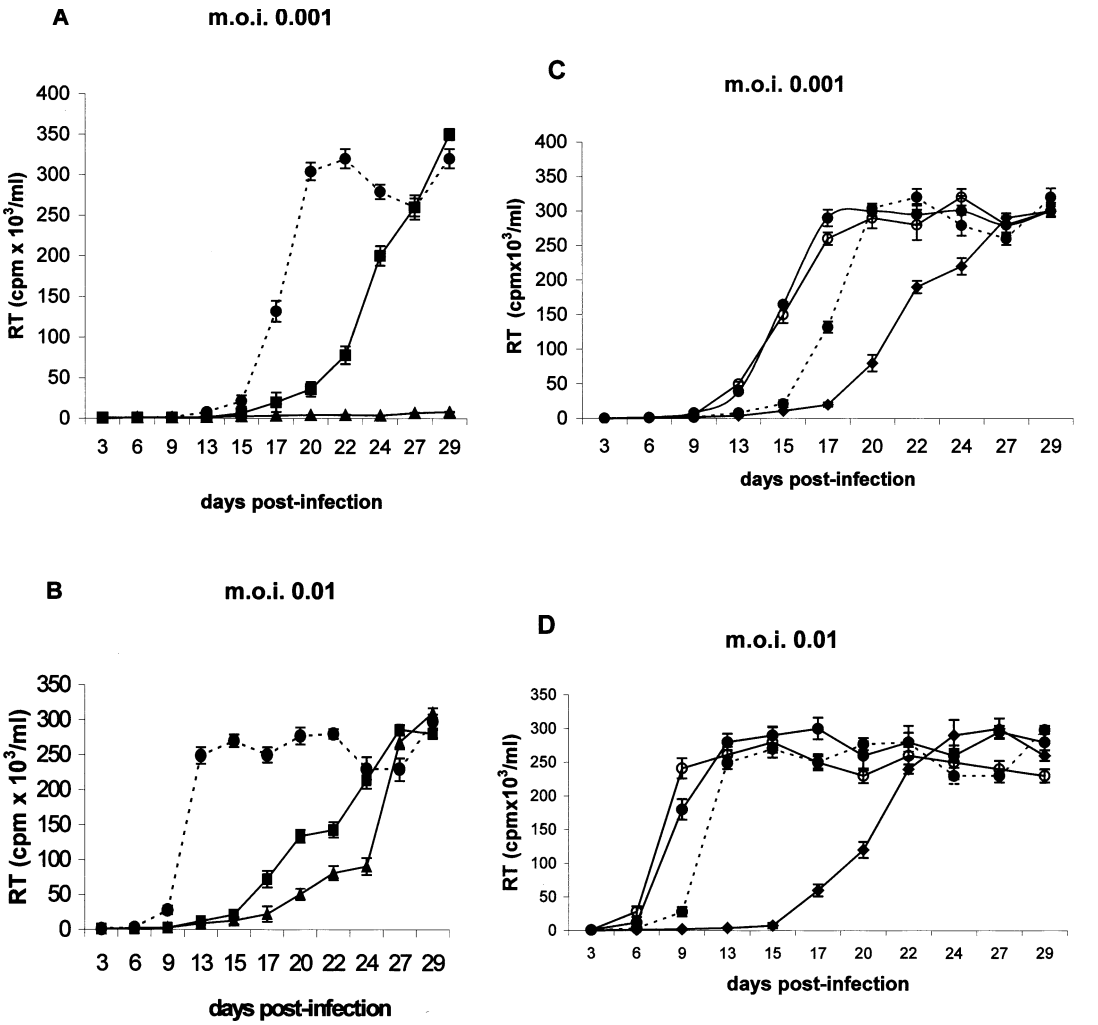


Fig. 5. Effect of therapeutic RNAs on HIV-1 replication. Cell culture supernatants were collected at designated time points after HIV-1 challenge of Jurkat cells transduced by retroviral vectors DC-tRNA-AT and DC-6tRNA-AT (J-DC-tRNA-AT and J-DC-6tRNA-AT, respectively, panels A and B), DC-AT-VAIα (5′–3′), DC-AT-VAIα (3′–5′) and DC-VAIα (J-DC-AT-VAIα (5′–3′), J-DC-AT-VAIα (3′–5′) and J-DC-VAIα, respectively, panels C and D), and monitored for reverse transcriptase (RT) activity.-- -●---, Jurkat; —■—, J-DC-tRNA-AT; —▲—, J-DC-6tRNA-AT; —●—, J-DC-AT-VAIα (5′–3′); —○—, J-DC-AT-VAIα (3′–5′); —◆—, J-DC-VAIα.

Table 1
Activity of the therapeutic genes in vitro: statistical evaluation

m.o.i.	Construct	*b*	*P*	Effect on HIV-1 replication
0.01	Control (Jurkat)	10.7		
	J-DC-tRNA-AT	21.8	0.000008	Inhibition
	J-DC-6tRNA-AT	26.9	0.000077	Inhibition
	J-DC-VAIα	20.1	0.000005	Inhibition
	J-DC-AT-VAIα (3′–5′)	7.2	0.007075	Enhancement
	J-DC-AT-VAIα (5′–3′)	8.5	0.032317	Enhancement
0.001	Control (Jurkat)	17.1		
	J-DC-tRNA-AT	24.5	0.000013	Inhibition
	J-DC-6tRNA-AT	NA	NA	Inhibition
	J-DC-VAIα	21.6	0.000001	Inhibition
	J-DC-AT-VAIα (3′–5′)	15.0	0.000082	Enhancement
	J-DC-AT-VAIα (5′–3′)	14.8	0.000007	Enhancement

Each experiment is defined by a m.o.i. level and by an indication concerning the construct (retroviral vector) employed. The transition centers are reported in column *b*, and expressed as days. An inhibition of HIV-1 replication is revealed by a longer time interval to the transition center with respect to the control, an enhancement by the opposite condition. NA: not applicable since HIV-1 replication was not observed; at m.o.i. = 0.001 the inhibition of HIV-1 replication by J-DC-6tRNA-AT was practically complete and the sigmoid growth model was not appropriate.

tagonist effect was therefore promoted by the association of the anti-*tat* tRNA and the anti-U5 hammerhead ribozyme when the two molecules were expressed by the same vector even in the form of individual transcripts. The molecular basis for this antagonistic effect is not clear yet. It could be hypothesized that the VAI structure interferes with the PKR-mediated antiviral activity that follows hybrid formation between the antisense anti-*tat*-tRNA and tat-mRNA in HIV-1-infected, DC-tRNA-AT transduced cells. The anti-HIV function of the VAIα ribozyme could also be sequestered in such VAI-PKR interaction (Kitajewski et al., 1986; O'Malley et al., 1994). These results suggest that alternative, indirect cellular mechanisms that warrant further studies may contribute to the inhibitory antiviral effect of RNA therapeutics. Kinetics analysis of HIV-1 gene expression might help in clarifying unexpected findings in experimental conditions where synergism/antagonism of different therapeutic combinations are looked for.

The co-expression of different antiviral genes could be promising for the treatment of HIV infection (Morgan and Walker, 1996; Duan et al., 1997; Fraisier et al., 1998), but to be successful a combined gene therapy approach should avoid the interference between the effector molecules. Peng and co-workers demonstrated that two effector RNAs were not able to inhibit HIV replication when expressed as a chimeric molecule (Peng et al., 1999) while displaying antiviral activity when expressed as separate transcripts. In addition, the design of the vector and the orientation of the transgenes could affect the therapeutic potency of the construct (Peng et al., 1996, 1997). Lisziewicz et al have recently shown the synergistic anti-HIV-1 effect of RNA decoys such as TAR and RRE when coexpressed as a single transcript (Lisziewicz et al., 2000). However, their results were obtained by transfection experiments and did not take into account levels of gene product. Thus, the real efficacy of these therapeutic molecules, when delivered in a conventional anti-HIV-1 gene therapy approach via transducing vectors, has not yet been established.

In conclusion, we demonstrate that a higher absolute steady state concentration of anti-*tat* antisense RNA is produced in cells transduced by a retroviral vector carrying multiple copies of the

therapeutic gene. The higher levels of antisense RNA in humanT-cells correlated with an increased inhibition of HIV-1 replication. HIV-1-specific CD4$^+$ cell lines modified by this genetic treatment could hence be proposed, in association with HAART, for adoptive immunotherapy of AIDS. Further studies are needed to find synergistic gene combinations able to abort early stages of lentiviral infection.

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