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Inhibition of HIV-1 Replication by an Anti-tat Hammerhead Ribozyme

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Tat is a virally expressed regulatory protein involved in the replication of HIV-1, the etiological agent of AIDS. To investigate the effect of tat inhibition on HIV replication, we constructed a retroviral vector to express an anti-tat hammerhead ribozyme as part of the 3′ untranslated region of β-galactosidase transcripts. Initial testing of this vector in tat-expressing COS-7 cells reduced tat activity by 85-95% as measured by tat-dependent CAT assays. Amphotropic and HIV-pseudotyped retroviral particles generated with this vector were used in HIV challenge experiments to determine the ability of this reagent to control HIV replication. $CD4^+$ peripheral blood lymphocytes (PBLs) stably transduced with this vector were subsequently challenged with HIV. These cells were able to resist HIV infection for up to 20 days as measured by cell death and reverse transcriptase activity. These data yield proof of principle that a pseudotyped retroviral vector can target and deliver a protective ribozyme to $CD4^+$ cells.

Several attempts have been made to block HIV replication using antisense and/or ribozyme reagents (1-4) however these studies have generated mixed results. Because of the catalytic nature of ribozymes (5) their use as gene therapy against viral diseases would appear to be superior to the use of antisense regents. In this regard Goodchild *et al.* (6) reported little ability of antisense reagents targeted to various regions of the HIV-1 tat sequence to inhibit viral replication. Subsequently, Lo *et al.* (4) compared the ability of anti-tat antisense and ribozyme reagents to protect Jurkat cells against HIV challenge. In this study they reported that antisense conferred greater resistance to HIV than a similar ribozyme reagent. On the other hand, Zhou *et al.* (3) showed that ribozymes targeting various HIV regions were superior to antisense in their ability to reduce HIV infection. To further test the efficacy of ribozymes to inhibit HIV replication, we generated a retroviral vector that expresses an anti-HIV tat hammerhead ribozyme as part of the 3′ untranslated region (UTR) of *E. coli* β-galactosidase (lacZ) transcripts.

Delivery of such reagents is a matter of much concern to those involved in their use. Of particular importance in AIDS is the delivery of therapeutic reagents to infected or potentially infected cells. In the case of HIV, these cells are $CD4^+$ (7,8). HIV and other retroviruses target cells through the interaction of viral envelope glycoproteins (env) with specific cell surface antigens (8). One of the most commonly used env genes in constructing retroviral vectors was derived from the Moloney Murine Leukemia virus (Mo-MLV). This gene encodes an amphotropic glycoprotein that binds to the ubiquitous cellular phosphate transporter (9) and therefore cannot be used to target specific cell populations. Alternatively, HIV particles target $CD4^+$ cells through expression of gp120, a viral glycoprotein that has high affinity for CD4 (8). In order to generate targeted retroviral particles carrying the anti-tat ribozyme, we attempted to generate a pseudotyped Mo-MLV virus utilizing the HIV env gene. The ability of this reagent both to target $CD4^+$ cells and to provide protection against HIV is discussed.

MATERIALS AND METHODS

Retroviral vector construction. A pGUC hammerhead ribozyme cleavage site (10) was identified within a 27 nucleotide sequence just downstream of the tat initiation codon of the HIV-1 molecular clone HXB2 (Los Alamos Data Bank, 1994). Based on this sequence, we designed and synthesized a hammerhead ribozyme to target HIV tat transcripts (Fig. 1A). The double stranded ribozyme was cloned into

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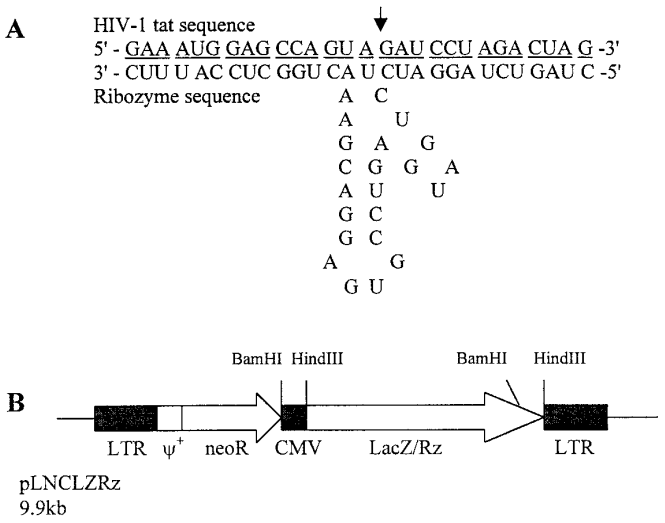

FIG. 1. Ribozyme sequence and retroviral vector structure. (A) HIV tat sequence and anti-tat ribozyme sequences are shown. The ribozyme substrate sequence is underlined. An arrow indicates the ribozyme cleavage site. (B) The retroviral vector pLNCLZRz is shown. The lacZ/ribozyme fusion gene was cloned into pLNCX as described.

the shuttle vector pNoTA (5 Prime → 3 Prime, Boulder, CO) by blunt end ligation. The 3kb *E. Coli* lacZ gene from the MLV retroviral vector plasmid p1744 was subsequently cloned into the *Eco*RI site just upstream of the inserted ribozyme. The lacZ/ribozyme fusion gene was removed with *Hind*III and cloned into the retroviral vector pLNCX to form pLNCLZRz (Fig. 1B). The components of this vector were verified by restriction analysis and direct sequencing.

Generation of retroviral particles. Amphotropic retroviral particles were generated by co-transfection of 293T cells using 5μg each of pLNCLZRz and pPAM3 based on the method of Pear *et al.* (11). Briefly, 293T cells, cultured in DMEM containing 10% FBS and 2mM L-glutamine, were split to approximately 70% confluence 24 hours prior to transfection by calcium phosphate precipitation (12). Transfected cells were washed with PBS at 24 hours post-transfection and re-fed. The following day, viral particles were collected in the producer cell culture fluid. The culture fluid was filtered (0.45μm), titered on NIH/3T3 cells, and stored at −70°C. NIH/3T3 cells were cultured in DMEM containing 10% newborn calf serum and 2mM L-glutamine. For titering virus, NIH/3T3 cells at approximately 50% confluence were incubated with equal volumes of producer culture fluid and NIH/3T3 medium. Polybrene was added at a concentration of 10μg/ml. Forty-eight hours later, the infected NIH/3T3 cells were washed, fixed, and stained with X-gal according to the method of Lim and Chae (13). The total number of blue cells per dish was used as a measure of viral titer.

To create a producer cell line capable of generating HIV-pseudotyped virions, NIH/3T3 cells were transfected with pgagpolgpt (14) by calcium phosphate precipitation. Stable transformants were selected for by growth in HXM medium (NIH/3T3 medium containing 15μg/ml hypoxanthine, 250μg/ml xanthine, and 25μg/ml mycophenolic acid). Several clones were expanded and assayed for reverse transcriptase (RT) activity using the RT-Direct (DuPont NEN, Boston, MA) assay kit. One clone having high RT activity was subsequently transfected with pDOLHIVenv (15,16). Stable transformants were selected for by the addition of 1mg/ml G418 to the HXM medium. Several clones were expanded and assayed for gp120 expression by dot blot analysis. Those having the highest levels of gp120 expression were transfected at a 1:10 ratio with pLNCLZRz and pRSVhygro (a plasmid that expresses the hygromycin resistance gene under control of the RSV LTR). Stable expression of pLNCLZRz was achieved by selection in HXM medium containing 200μg/ml hygromycin. Hygromycin-resistant cells were expanded for use as NIH/3T3 gp120 producer cells. Viral titers were determined as above using HeLa CD4 cells (17,18) cultured in DMEM containing 10% FBS and 2mM L-glutamine.

Transduction of CD4$^+$ PBLs with a pseudotyped retrovirus. Fifty milliliters of fresh blood from healthy donors were collected by venipuncture. Leukocytes were isolated on Histopaque 1.077 (Sigma, St. Louis, MO), washed with PBS, and incubated in PBS/1mM EDTA/0.5% human γ-globulin for 15 minutes at 25°C. CD4$^+$ peripheral blood lymphocytes (PBLs) were subsequently isolated using an anti-CD4 antibody coated tissue culture flask (Applied Immune Sciences, Santa Clara, CA) according to the manufacture's protocol. Isolated CD4$^+$ PBLs were stimulated to proliferate by the addition of 100ng/ml phorbol 12-myrsitate 13- acetate (PMA) and cultured in DMEM/F12 containing 15% FBS, 15mM HEPES, 10mM sodium pyruvate, and 2mM L-glutamine supplemented with 1000U/ml human rIL-2 (LCM). Expanded CD4$^+$ PBLs were incubated with equal volumes of filtered culture fluid containing HIV-pesudotyped virions and lymphocyte culture medium (LCM). Polybrene was added at a concentration of 4μg/ml. After 48 hours, the cells were washed with PBS and re-fed. Stable expression of the retroviral construct was obtained by selection in 1-4mg/ml G418. Clonal populations were expanded and stored until needed in HIV challenge studies.

HIV challenge and RT assays. CD4$^+$ PBLs stably expressing the anti-tat ribozyme construct were challenged with HIV-1 (HTLV-IIIB) (19,20) to determine ribozyme effectiveness against HIV replication. Non-transduced and ribozyme-transduced cells were plated at 1×10^6 cells each after which HIV-1 was added at a MOI of one. Identical cultures without HIV were prepared as controls. Following overnight incubation, cells were washed with PBS and re-fed with fresh LCM. Each culture was monitored over a 21 day period for RT activity and cell death by trypan blue exclusion. Reverse transcriptase activity was determined using the RT-Direct assay kit. Briefly, 200μl of culture fluid was removed and centrifuged to remove cell debris. Any viral particles present were pelleted by ultracentrifugation at 200,000×g for one hour and resuspended in 30μl of lysis buffer. Lysates were tested for RT activity by incubation with the supplied template, primer and all four dNTPs to generate a cDNA copy of the original template. Following hydrolysis of the template, each reaction was incubated with a cDNA-specific biotinylated antibody and a peroxidase-conjugated detector in a streptavidine coated plate. The plate was washed, TMB was added as a substrate, and the amount of cDNA present was quantified spectrophotometrically at 650nm. The quantity of cDNA, measured as fmol cDNA/ml, was used as an indirect indicator of reverse transcriptase activity.

TABLE 1

Retroviral Titers Generated Using Amphotropic and HIV-Pseudotyped Packaging Plasmids

Amphotropic LNCLZRZ	HIV-pseudotyped LNCLZRz	HIV-pseudotyped control LNCLZ
1.7×10^4	4.5×10^2	6.6×10^3
4.1×10^4	1.5×10^2	0*
4.9×10^4	1.5×10^2	7.5×10^2

Note. Amphotropic titers as measured on NIH/3T3 cells were consistently on the order of 10^4 particles/ml of culture fluid. HIV-pseudotyped retroviral titers as measured on HeLa CD4+ cells were generated using pLNCLZRz and pLNCLZ (Rz-). HIV-pseudotyped titers were ranged from 10^2 for ribozyme positive cultures to 10^3 for ribozyme negative cultures. One sample (*) was discarded due to poor initial transfection efficiency.

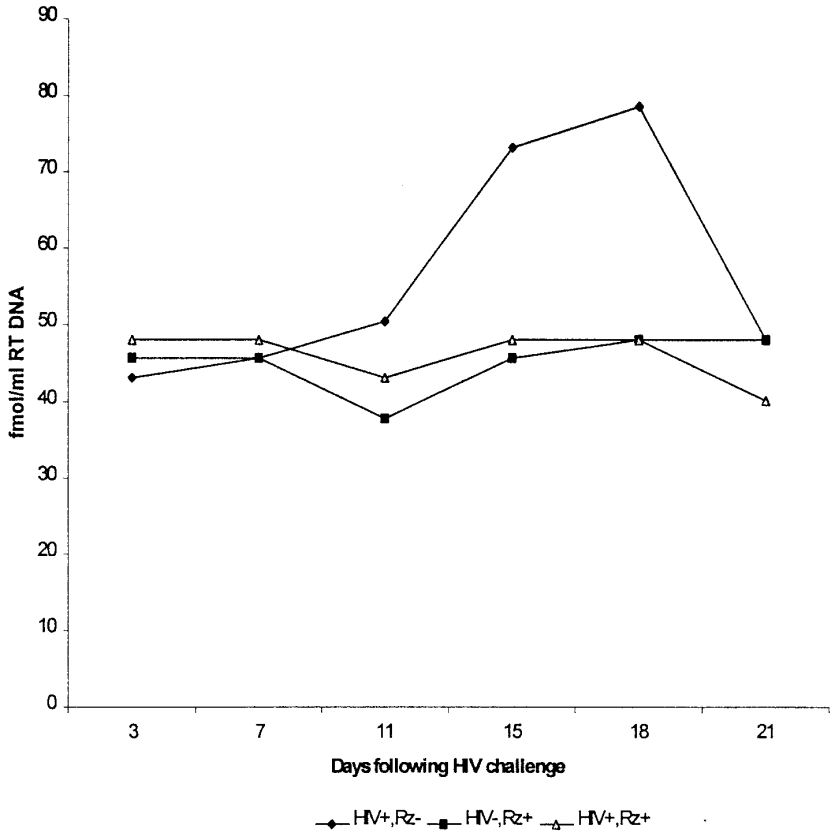

FIG. 2. Anti-tat ribozyme effect on HIV reverse transcriptase (RT) activity in HIV-challenged $CD4^+$ peripheral blood T cells. RT activity was measured in cell culture lysates as described at six times following initial HIV challenge.

RESULTS

Generation of pseudotyped retroviral vectors. NIH/3T3 cells, transfected with pgagpolgpt, were selected for stable integration by growth in HXM medium. Twenty-four HXM-resistant clones were analyzed for RT activity as expressed from the transfected construct pgagpolgpt. High levels of RT activity was detected in one clone which was subsequently transfected with pDOLHIVenv and selected for stable expression using G418. Forty-two G418-resistant clones were tested for gp120 expression by immunoenzyme dot blot assays of cell lysates and/or anti-gp120 immunofluorescence of fixed cells. Three highly positive clones were co-transfected with pLNCLZRz and pRSVhygro followed by hygromycin selection of stable lines. Several hygromycin-resistant clones were expanded for use as retroviral producer cells. HIV-pseudotyped retroviral particles were harvested in the culture fluid of the NIH/3T3 gp120 producer cells. The harvested culture fluid was filtered (0.45μm), titered on HeLa CD4 cells, and stored at -70°C until needed. A comparison of the titers obtained showed that the pseudotyped virions were consistently generated at significantly lower titers with respect to amphotropic virions generated in 293T cells (Table 1).

HIV challenge of CD4+ PBLs. CD4+ PBLs were infected with HIV-pseudotyped virions expressing the anti-tat ribozyme and selected for stable expression by growth in LCM supplemented with G418. Stable clones were frozen until needed. For HIV challenge studies, both ribozyme-expressing and non-expressing $CD4^+$ PBLs were challenged with HIV-1 to determine the ribozyme's ability to inhibit or reduce HIV replication. Culture fluid samples taken from these cells were analyzed for RT activity as described. Reverse transcriptase activity in ribozyme negative cells progressively increased from background at day one to 78 fmol/day at day 18 before excessive cell death resulted in a return to background levels by day 21(Figure 2). Alternatively, RT activity in non-infected cells and ribozyme-expressing infected cells showed similar patterns to day 21.

DISCUSSION

The results of this study are twofold. These data suggest that HIV replication in infected cells can be reduced by utilizing a hammerhead ribozyme directed against sequences proximal to the HIV-1 tat translation initiation site. Secondly, these data show that HIV-

pseudotyped retroviral particles have the potential to be used as vectors to deliver therapeutic genes to targeted cells. In this study however, HIV-pseudotyped virus titers were consistently lower than those generated by conventional methods using an amphotropic packaging plasmid (Table 1). While part of the reason for this may be that the HIV gp120 is not compatible with the Mo-MLV packaging machinery, it is possible that this effect may also be due to ribozyme expression within the producer cells. While ribozyme expression in HIV infected cells targets tat mRNA, the ribozyme likely targets pDOLHIV mRNAs which contain the target sequence in producer cells used to generate HIV-pseudotyped virions. In producer cells generating amphotropic virions, there is no such target available, resulting in higher titers.

In an investigation similar to this, $CD4^+$ PBLs were transduced with a retrovirus containing a ribozyme targeted to tat mRNA (21). This study reported a 70% reduction in p24 antigen synthesis up to 12 days post-infection with HIV. Vandendriessche *et al.* (22) transduced human $CD4^+$ lymphocytes with four different anti-HIV vectors, including an antisense vector directed to the HIV-1 genes tat and rev. These workers showed that all four vectors were able to inhibit HIV replication following infection with four different clinical isolates. Similarly, our data show that expression of an anti-tat ribozyme can inhibit HIV replication in $CD4^+$ PBLs. In the present study, ribozyme transduced $CD4^+$ PBLs showed reduced levels of HIV replication by 60-70% (inhibition at the peak of RT production in non-protected cells). This protection was evident between day 11 and 18 post-challenge with HIV (Fig. 2).

Taken together, this study shows the utility on an anti-tat ribozyme to significantly reduce HIV replication within a population of infected cells. One problem that continues to plague the use of these reagents is their short-term effectiveness. Our study did not address this issue past 21 days. It is likely that the use of these reagents would be best employed in conjunction with other therapies in order to enhance the overall effectiveness of the combination. While such strategies may not completely eliminate HIV infection, such combination therapies may greatly increase $CD4^+$ lymphocyte survival.

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