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Inhibition of CCR5-Dependent HIV-1 Infection by Hairpin Ribozyme Gene Therapy against CC-Chemokine Receptor 5

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CCR-5 is a major cellular coreceptor for R5 strains of HIV-1. Individuals carrying a homozygous 32-base-pair deletion in this gene are apparently healthy and are relatively resistant to HIV-1 infection. Since CCR5 appears to be dispensable for the host, but important for initial HIV-1 infection, CCR5 mRNA is an excellent therapeutic target for inhibiting HIV-1 replication via ribozyme knockout. We report here that hairpin ribozymes are able to reduce cellular CCR5 mRNA and cell surface CCR5 when stably introduced into PM1 cells by transduction with recombinant adenoassociated viral vector. The ribozymes effectively protect the cells from infection by R5 HIV-1 strains or non-syncytium-inducing clinical isolates commensurate with a reduction in CCR5 mRNA. These results suggest a novel gene therapy approach to preventing or slowing the disease progression of HIV-1 infection.

Entry of HIV type 1 (HIV-1) into human $CD4^+$ cells requires certain chemokine receptors expressed on the target cell surface such as the coreceptor for HIV-1 envelope glycoproteins (env) (for reviews see Berger, 1997; Doranz *et al.,* 1997; Moor *et al.,* 1997). The coreceptor interacts with the V3 loop of gp120 of env which becomes exposed following the initial binding of env to CD4 on the target cell surface (Wu *et al.,* 1996).

CCR5 and CXCR4 are two major coreceptors used by macrophage tropic (M-tropic or R5) and T cell line tropic (T-tropic or X4) HIV-1 strains, respectively (for review see Berger, 1997; Doranz *et al.,* 1997; Moor *et al.,* 1997). Both of them can also be used by duotropic strains. In addition to CCR5 and CXCR4, other closely related chemokine receptors such as CCR1, CCR2b, CCR3, STRL33/Bonzo, and BOB may also serve as coreceptors for some HIV-1 strains (Deng *et al.,* 1997; Liao *et al.,* 1997). Among these coreceptors, CCR5 is often used by HIV-1 to establish initial infection. The majority of individuals who lack a functional CCR5 due to a homozygous 32-base-pair deletion in the CCR5 gene appear relatively resistant to HIV infection (Liu *et al.,* 1996; Paxton *et al.,* 1996; Samson *et al.,* 1996a). Individuals who are heterozygous for the CCR5 delta 32 mutation have a lower level of CCR5 expression than the homozygotes carrying the wild-type CCR5 gene (Wu *et al.,* 1997). Although there is no indication that the heterozygotes are less susceptible to HIV-1, some evidence shows that the disease progression in HIV-1-infected heterozygotes is often slower than that of the infected individuals homozygous for the wild-type CCR5 gene (Buseyne *et al.,* 1998; Eugen-Olsen *et al.,* 1997; Paxton *et al.,* 1998a,b). Taken together, these observations suggest that elimination or even partial reduction of CCR5 may protect individuals from HIV-1 infection or slow down disease progression. Since CCR5 is dispensable for normal cellular and physiological function (Liu *et al.,* 1996; Paxton, 1996; Samson *et al.,* 1996a), it could be an attractive target for ribozyme-mediated gene therapy.

Recently, several different approaches have been used to inhibit the cellular expression of CCR5 and CXCR4 (Bai *et al.,* 1998; Carroll *et al.,* 1997; Chen *et al.,* 1997; Yang *et al.,* 1998). One of them employs anti-CCR5 ribozymes. It was reported that the addition of synthetic hammerhead ribozymes was able to inhibit CCR5 expression when both CCR5 and the ribozymes were transiently transfected into HeLa or HEK 293 cells (Goila and Banerjea, 1998; Gonzalez *et al.,* 1998). Transient transfection of synthetic ribozymes, however, is unlikely to be of therapeutic benefit, since RNA is highly unstable in cells. Furthermore, these studies did not determine whether anti-CCR5 ribozymes were able to reduce endogenous CCR5 and, if so, whether such a reduction in CCR5 could render the cells resistant to HIV-1 infection. These two questions are important to determine whether anti-coreceptor ribozymes can be used in gene therapies against HIV-1 infection.

In this report, we describe a ribozyme gene therapy approach to HIV treatment. Intracellular delivery and

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TABLE 1

Kinetic Parameters of Anti-CCR5 Ribozymes

Ribozyme	K_m (nM)	kcat	kcat/K_m (10^4 M^{-1} min^{-1})
R5-14	8.9	0.065/min	730
R5-150	15	0.16/min	1067
R5-392	9.7	0.15/min	1598
R5-670	17	0.12/min	676
R5-897	65	0.12/min	184
Native ribozyme	17	0.63/min	3705

Note. The ribozyme concentration was 4 nM and the substrate concentration ranged from 4 to 250 nM.

expression of catalytically active hairpin ribozyme genes, directed against CCR5, are achieved using recombinant adenoassociated virus (rAAV) vectors. Constitutive intracellular production of these ribozymes resulted in a reduction of endogenous CCR5 mRNA and protein in the vector-transduced cells. More importantly, introduction of these ribozyme genes substantially reduced the susceptibility of the cells to infection by R5 HIV-1 but not X4 HIV-1 strains. Therefore, these anti-CCR5 ribozyme genes may be utilized as an effective gene therapy, perhaps in combination with ribozyme genes that target the viral RNA directly (for reviews see Welch *et al.,* 1996a; Yu *et al.,* 1994) or in combination with current antiviral drugs therapies, as an optimal treatment for HIV infection.

RESULTS

In vitro kinetic characterization of anti-CCR5 hairpin ribozymes

Based on the human CCR5 mRNA sequence, 53-nucleotide-long hairpin ribozymes which contain 4 base and 8 base-target recognition sequences were synthesized according to the previously characterized hairpin ribozyme motif (Hampel, 1998). Initially, eight distinct candidate ribozymes were screened for cleavage activity *in vitro* against their respective 19-nucleotide CCR5 RNA substrates. Preliminary experiments with these ribozymes indicated that five of eight showed substantial cleavage of their respective target RNAs *in vitro* (data not shown). These five ribozymes, designated R5-14, R5-150, R5-392, R5-670, and R5-897 (the number indicates the cleavage sites in CCR5 mRNA relative to the AUG protein translation start site (Combadiere *et al.,* 1995; Samson *et al.,* 1996b), were further characterized for their kinetic properties. The catalytic properties of these ribozymes varied widely (Table 1), but are comparable to that of the previously tested anti-HIV, HBV, and HCV ribozymes, which have sufficient potency to produce antiviral activity (Welch *et al.,* 1996a,b, 1997).

In vivo expression of CCR5 ribozyme genes

Three of the five catalytically characterized ribozymes were selected for further analysis by ribozyme gene therapy *in vivo.* The choice of ribozyme was based on (1) their ability to cleave their respective target RNAs *in vitro* (Table 1) and (2) their specificity for CCR5, as determined by the uniqueness of their target recognition domains (at least three base pair differences compared with all known human sequences). For *in vivo* expression of the hairpin ribozymes, double-stranded oligonucleotides encoding ribozymes R5-14, R5-392, and R5-897, respectively, were cloned into an adenoassociated virus (AAV)-based vector plasmid, pAMFT, directly downstream of a tRNA valine promoter (Fig. 1), such that the ribozyme synthesized by RNA polymerase III would be processed as cellular tRNA. The plasmids were then used to generate rAAV vector particles capable of delivering each individual ribozyme gene and the neo-aminoglycoside phosphotransferase gene.

The human T cell line, PM1, was then transduced with the rAAV vectors and selected in G418-containing medium. This cell line expresses both CXCR4 and CCR5 and is therefore susceptible to infection by both CXCR4-specific and CCR5-specific HIV-1 strains (Lusso *et al.,* 1995). The use of this cell line facilitated the test of the specificity of the ribozymes, since ribozymes directed specifically against CCR5 should inhibit R5 HIV-1 strains, but not X4 HIV-1 strains that require CXCR4 for viral entry. As controls, PM1 cells were also transduced with rAAV vectors encoding the corresponding "disabled" ribozymes, whose catalytic activity had been inactivated through mutation of three bases known to be required for ribozyme cleavage activity (Welch *et al.,* 1997). After stably transduced PM1 cells were selected, the chromosome-integrated inserts were verified by PCR and DNA sequencing. The expression of ribozymes was also confirmed by reverse transcriptase (RT) PCR (data not shown).

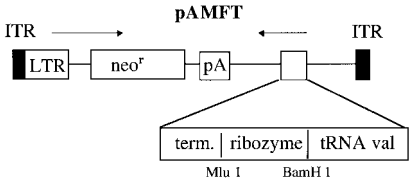

FIG. 1. Structure of the rAAV ribozyme expression plasmid pAMFT. pAMFT is constructed on the basis of the retroviral vector plasmid pLNL6. The diagram shows only the section between the AAV inverted terminal repeats (ITR). The selection marker neo-aminoglycoside phosphotransferase (neo^r) coding region is linked to an upstream long terminal repeat (LTR) promoter derived from the Moloney murine leukemia virus genome. DNA encoding CCR5 ribozymes is placed downstream of a tRNA valine promoter. pA, SV40 polyadenylation site; term, pol III termination site. The arrow shows the direction of transcription.

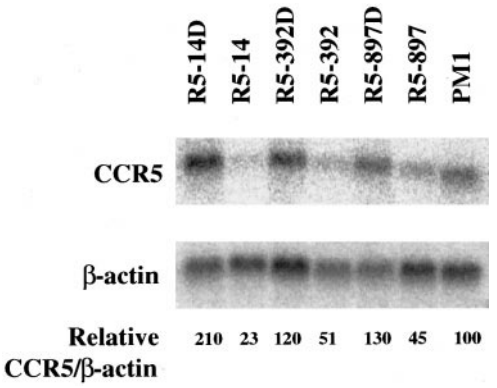

FIG. 2. Northern blot analysis of the cellular CCR5 mRNA. A Northern blot of mRNA isolated from parental PM1 cells and the cells transduced to express CCR5 ribozymes or their disabled counterparts (D) was hybridized with CCR5-specific probes and then with β-actin-specific probes for normalization of RNA loaded. The relative CCR5/β-actin is expressed as the relative ratio of the phosphorimage density of CCR5 mRNA to β-actin mRNA while the ratio of the parental PM1 cell mRNAs is defined as 100.

In vivo cleavage of CCR5 mRNA by CCR5 ribozymes results in a decrease in CCR5 expression

To determine whether *in vivo* expressed CCR5 ribozymes were able to cleave CCR5 mRNA, total mRNA was extracted from the parental PM1 cells and the cells transduced to express CCR5 ribozymes. The level of CCR5 mRNA expression was determined using Northern blot analysis with ^{32}P-labeled CCR5 cDNA fragments as the hybridization probe. The CCR5 mRNA was then normalized to the cellular β-actin mRNA level, which was used as an internal standard. As shown in Fig. 2, the mRNA level in the cells expressing R5-14 was reduced by 77% of that in the parental PM1 cells. In the cells expressing R5-392 and R5-897, CCR5 mRNA decreased at least 50%, whereas in the cells expressing disabled ribozymes, there was no decrease in CCR5 mRNA expression. The differences in mRNA level observed for different ribozymes do not seem to correlate with enzymatic activity. This may reflect variable steric availability of the specific target sites by each ribozyme, due to RNA folding or, more likely, RNA binding proteins *in vivo.*

Next, the cell surface CCR5 was measured to determine whether the ribozyme-mediated reduction in CCR5 mRNA would yield meaningful changes in CCR5 surface expression. The PE-conjugated anti-CCR5 monoclonal antibody 2D7/CCR5 from PharMingen was used to label the CCR5 on R5-14 cells. Surface expression is detectable on 18% of PMI cells, which is in agreement with previously published results of 20% positive staining for PM1 cells (Wu *et al.,* 1997). In the case of the R5-14-expressing cells, about 80% reduction in the surface CCR5 protein was consistently observed (Fig. 3). Thus the reduction in the cell surface CCR5 correlates with the decrease in the cellular CCR5 mRNA.

Inhibition of CCR5-dependent cell fusion by *in vivo* expression of CCR5 ribozymes

Chemokine receptors facilitate entry of HIV-1 into cells by allowing fusion of the HIV env-containing viral particles to the target cell membrane containing CD4 and the chemokine receptor. To test the effect of CCR5 ribozymes on the coreceptor-dependent fusion event, an experimental cell/cell fusion assay was used that mimics many of the characteristics of virus/cell fusion. Fusion between the cells expressing HIV-1 env and the target cells expressing CD4 and the coreceptors requires a specific match between the type of HIV-1 env and the coreceptors (for reviews see Berger, 1997; Doranz *et al.,* 1997; Moor *et al.,* 1997). Thus the cell fusion assay can be employed to determine the functional protein level of CCR5 on the cell surface.

To make HIV-1 env and T7 RNA polymerase-expressing cells, HeLa cells were coinfected with two vaccinia recombinants, vTF7-3 and either vCB43 or vSC60. vTF7-3 contains a bacteriophage RNA polymerase expression cassette under the control of a vaccinia early/later promoter. vCB43 contains an expression cassette of R5 HIV-1 Ba-L env. vSC60 contains a expression cassette of X4 HIV-1 IIIB env. The resulting HeLa cells express high levels of HIV-1 env on the cell surface and T7 RNA polymerase in the cytoplasm. The fusion partner PM1 cells were infected with vaccinia recombinant vCB21R-

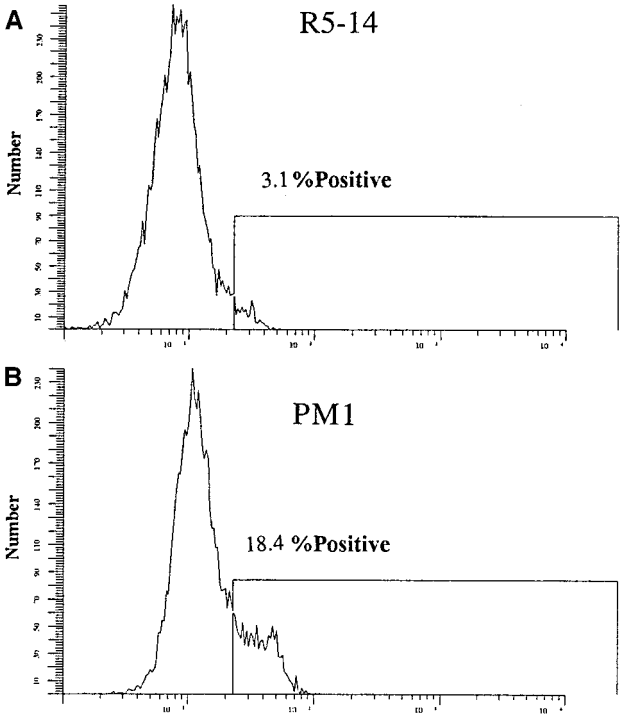

FIG. 3. FACS analysis of the cell surface CCR5. The cells were incubated with PE-conjugated mouse anti-CCR5 monoclonal antibody 2D7/CCR5 and then analyzed on a FACScan flow cytometer, using an isotype control to set the gating. (A) The PM1 cells expressing the ribozyme R5-14. (B) Parental PM1 cells.

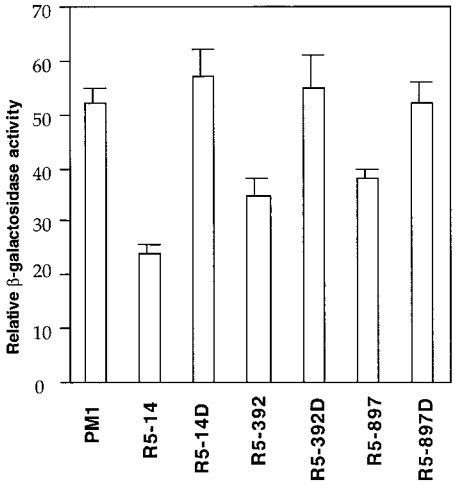

FIG. 4. HIV-1 env-mediated cell fusion. HeLa cells infected with vaccinia recombinants to express T7 RNA polymerase together with either Ba-L or IIIB env were mixed with the transduced or parental PM1 cells overexpressing CD4. Cell fusion was analyzed based on the relative β-galactosidase activity. The transduced PM1 cells were expressing each of the active ribozymes R5-14, R5-392, and R5-897 and the disabled counterparts (D). Error bars show the standard deviation of two measurements.

lacZ and vCB3. vCB21R-lacZ contains a lacZ gene under the control of a T7 promoter. vCB3 contains a expression cassette of CD4 under the control of a vaccinia early/later promoter. The resulting PM1 cells overexpress CD4 molecules on the cell surface. Overexpression is necessary to stimulate the otherwise inefficient process of cell fusion. After these two populations of vaccinia recombinant-infected cells are mixed together, if cell fusion occurs, T7 RNA polymerase in HeLa cells will transcribe the lacZ gene in the PM1 cells to produce β-galactosidase. The degree of cell fusion thus can be quantified according to the relative activity of β-galactosidase.

Since both CD4 and HIV-1 env are overexpressed in this cell fusion system, the only limiting factor for cell fusion would be expected to be the coreceptor. The β-galactosidase activity should correlate with the level of the coreceptor on the cell surface. In order to compare the CCR5-dependent cell fusion between different samples, the CCR5-dependent fusion data in Fig. 4 are normalized to the level of the CXCR4-dependent cell fusion. For all samples we tested, expression of hairpin ribozymes did not show any inhibitory effect on the CXCR4-dependent cell fusion (data not shown). As shown in Fig. 4, the fusion between the PM1 cells expressing R5-14 and HeLa cells expressing Ba-L env is about 50% of that between the parental PM1 cell and the HeLa cells. A decrease in cell fusion was also observed for the PM1 cells expressing R5-392 and R5-897, but the reduction is only about 30%. PM1 cells expressing disabled ribozymes R5-14D, R5-392D, and R5-897D had no significant change in cell fusion. These data indicate that the ribozyme-mediated decrease in surface CCR-5 is sufficient to inhibit HIV-1 env/CD4-dependent membrane fusion. Since this cell/cell fusion model assay only partially resembles the more facile virus/cell fusion process, the data should not be taken as a quantitative prediction of anti-viral effects.

Inhibition of CCR5-dependent HIV-1 infection by intracellular expression of CCR5 ribozyme

To determine whether intracellular CCR5 ribozymes were able to specifically protect PM1 cells from R5 HIV-1 infection, the transduced cells were challenged with R5 HIV-1 strain Ba-L at an m.o.i. of 0.02. The progress of the resultant infection was followed by measuring p24 capsid protein in the medium. The data in Fig. 5 indicate that all three ribozymes, R5-14, R5-392, and R5-897, were capable of substantially inhibiting HIV replication throughout the 15-day experiment. To test the specificity of the anti-viral effect, ribozyme gene-transduced PM1 cells were infected with either X4 HIV-1 strain IIIB or an R5 HIV strain Ba-L. The virion production was measured on day 15 postinfection. The cells transduced with ribozyme genes R5-14, R5-392, and R5-897 were all highly resistant to Ba-L infection (Fig. 5A). Virion production during 15 days postinfection was inhibited by greater than 99% for all three ribozymes, which is comparable to the inhibition with 100 nM RANTES, a natural ligand of CCR5 (Fig. 5A). The inhibition of viral replication by anti-CCR5 ribozymes was highly specific, since the same cells that were protected from R5 HIV stain Bal-1 infection retained susceptibility to X4 HIV strain IIIB, which uses CXCR4 as the coreceptor instead of CCR5 (Figs. 5A and 5B). Furthermore, the data in Fig. 5 indicate that ribozyme enzymatic cleavage activity was absolutely required for the observed inhibition of Bal-1 HIV replication, since cells transduced with catalytically "disabled" ribozymes were fully susceptible to infection by both Ba-L and IIIB. Thus, an antisense effect on the ribozyme target recognition domain alone cannot account for the observed results, as the disabled ribozymes lack enzymatic activity, but maintain the complementary target recognition domain.

In order to determine whether anti-CCR5 ribozymes can protect cells against uncloned HIV-1 primary isolates (quasispecies), a non-syncytium-inducing clinical isolate (076-0326) from an infected patient was used to challenge the PM1 cells expressing R5-14. The R5-14 transduced cells were chosen because it showed greatest apparent reduction in levels of mRNA for CCR5 by Northern blotting (Fig. 2). As shown in Fig. 5C, R5-14 showed significant antiviral activity (>90% reduction of p24) against the clinical isolate compared with the disabled R5-14, indicating that CCR5 ribozymes are effective at inhibiting replication of HIV quasispecies.

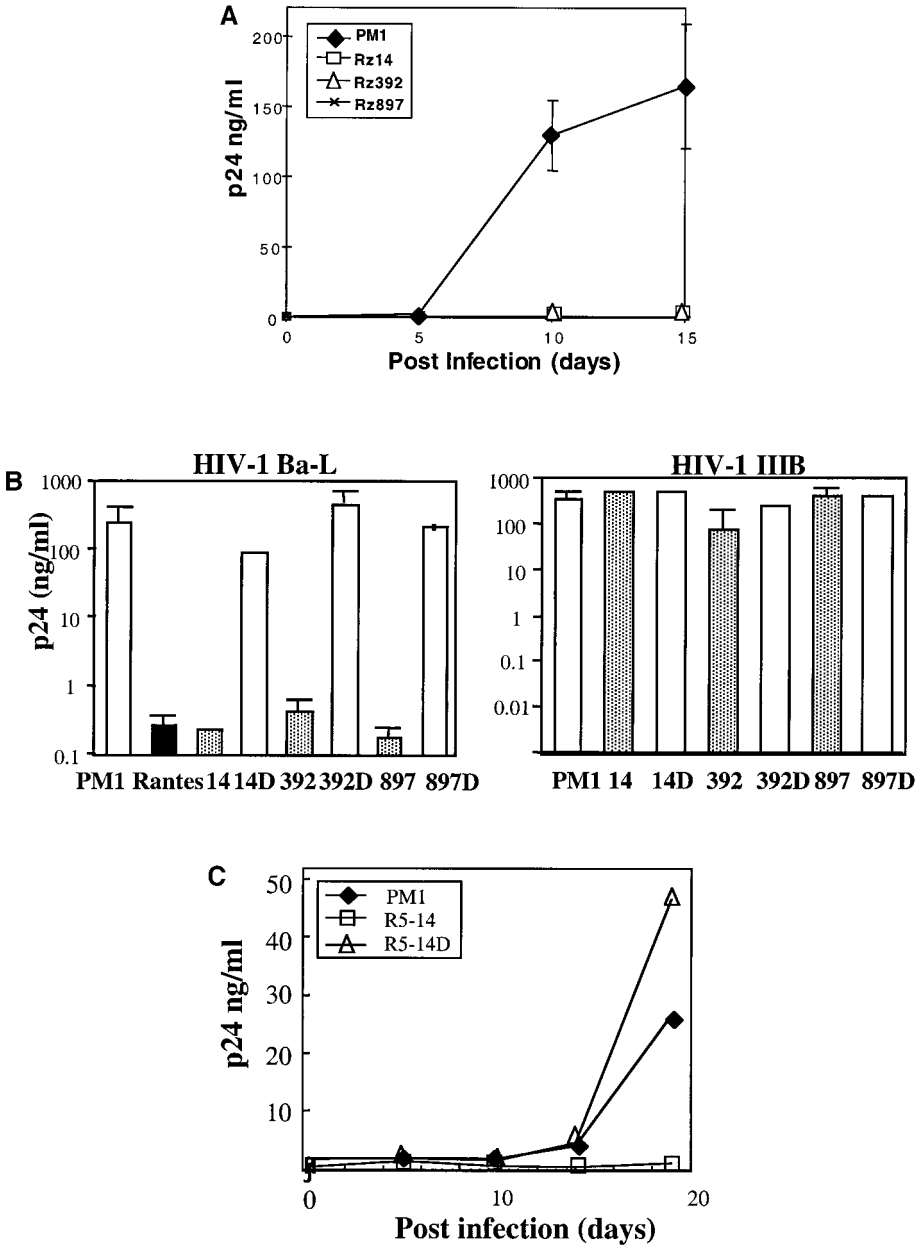

FIG. 5. (A). Time course for HIV Bal-1 replication. The PM1 cells transduced to express CCR5-ribozyme R5-14, R5-392, and R5-897 were incubated with the HIV-1 Ba-L at an m.o.i. of 0.02 for 2 h. Viral production was determined by a p24 antigen assay as a function of time postinfection. The parental PM1 cells were used as a negative control. Error bars show the standard deviation of triplicate measurements. (B). HIV-1 challenge of CCR5 ribozyme-expressing PM1 cells. Target cells were incubated with the virus at an m.o.i. of 0.02 for 2 h. Cells transduced with each disabled ribozyme (D) are represented by white bars, and cells transduced with each active ribozyme are represented by shaded bars. Viral production was determined by a p24 antigen assay on day 15 postinfection. (Left) R5 HIV-1 Ba-L was used. RANTES (100 nM) was used as a control (black bar) which was present during infection and postinfection. (Right) X4 HIV-1 IIIB was used. In each case, untransduced PM1 cells were used as a negative control. (C). Challenge of PM1 and the R5-14-expressing PM1 cells by HIV-1 quasispecies. The target cells were incubated with non-syncytium-inducing HIV-1 quasispieces (076-0326) isolated from a infected patient at an m.o.i. of 0.01 for 2 h. Viral production was determined by a p24 antigen assay as a function of time postinfection.

DISCUSSION

In this study, we demonstrated that intracellular expression of CCR5 ribozymes is able to specifically reduce the cellular CCR5 mRNA level, leading to a decrease in cell surface CCR5. As a result of reduction of CCR5 on the cell surface, CD4/HIV env-mediated membrane fusion is inhibited and the cells become resistant to R5 HIV-1 but not X4 HIV-1 infection. Such a high specificity makes CCR5 ribozymes suitable for use in gene therapies against HIV-1 infection. Moreover, hairpin ribozyme is a small RNA molecule. When stably expressed in cells, CCR5 ribozyme does not have any

deleterious effects. If gene transfer technologies allow CCR5 ribozyme to be expressed in a sufficiently large population of $CD4^+$-T lymphocytes in humans, these cells will become resistant to R5 HIV-1 and thus be able activate cytotoxic T lymphocytes and B lymphocytes upon HIV infection. The resulting antiviral immune responses could slow down the disease progression.

According to our results, the susceptibility of the cells expressing CCR5 ribozyme to R5 HIV-1 may not linearly correlate with the amount of residual CCR5 expression. Based on expression data (i.e., Northern blot and cell fusion assays), compared with our standard HIV challenge experiments, the differences in CCR5 among cell lines were not reflected in the degree of infectability. We hypothesize that there may be a threshold of CCR5 and CD4 on the target cell surface which is necessary for efficient entry of HIV-1, as has been previously shown for CXCR4 and CD4 (Dimitrov *et al.,* 1999; Xiao *et al.,* 1999). Indeed, there is some evidence showing that CD4 constitutively interacts with CCR5 on the cell surface of primary $CD4^+$T cells and macrophages that are known to be susceptible to the R5 HIV-1 and that CCR5 that does not associate with CD4 cannot be efficiently used by HIV (Divitrov *et al.,* 1999; Xiao *et al.,* 1999). This could explain why we see effective inhibition of HIV-1 infection, under our standard antiviral assay conditions, for all three of the CCR5 ribozymes despite the observed differences in the other measurements of their relative activities. If a threshold effect exists *in vivo,* it would imply that complete knockdown of the coreceptor may not be required for efficient inhibition of HIV infection of ribozyme transduced $CD4^+$ cells.

A reduction of cellular CCR5 expression associated anti-viral effect can also be inferred from HIV-1-infected individuals heterozygous for the CCR5 deletion. These individuals have decreased CCR5 on their lymphocytes and have slower disease progression compared with homozygotes carrying wild-type CCR5 (Buseyne *et al.,* 1998; Eugen-Olsen *et al.,* 1997; Paxton *et al.,* 1998a,b). In addition, the level of CCR5 expression has been shown to correlate with infectability of PBMCs by macrophage tropic HIV-1 *in vitro* (Wu *et al.,* 1997). Thus this further suggests that CCR5 ribozymes have the potential to be used as antiviral drugs against HIV-1.

A recognized concern with blocking the entry of R5 HIV-1 is the potential of providing selective pressure on HIV-1 to use the other major coreceptor, CXCR4, which is associated with strains of HIV-1 present during the occurrence of AIDS (Este *et al.,* 1999). In order to address this concern, we are currently developing anti-CXCR4 ribozymes under the control of T-cell-specific promoters, which can be delivered together with the anti-CCR5 ribozymes into human T cells or hematopoietic stem/progenitor cells before they differentiate into multilineage progeny cells *in vivo.* It is also important to note that it was recently shown by Chen and colleagues (Bai *et al.,* 1998; Chen *et al.,* 1997) that the CXCR4 could be knocked out by an intrakine gene therapy strategy in primary lymphocytes with the cells retaining normal biological functions. The present data suggest that a combination of anti-coreceptor ribozymes and anti-HIV ribozymes may offer one of the most promising approaches for gene therapy against HIV infection. One potential problem underlying the recent clinical success with antiviral drug regimes against HIV is the generation of resistant mutants, which may ultimately render triple drug therapy ineffective. The present anti-CCR5 ribozyme gene therapy strategy is aimed at the conserved regions of a cellular gene, which does not have the mutagenic potential of the HIV viral genome. Furthermore, the anti-CCR5 ribozyme approach is aimed at the entry level of HIV prior to the start of the replication cycle, which should limit the possibility of HIV drug-resistant mutations arising.

MATERIALS AND METHODS

Viruses and cells

HIV stocks of the IIIB strain were produced in H9 cell (ATCC HTB-176) cultures and the Bal-1 strain was produced in primary macrophage cultures. Clinical isolates were kindly provided by Dr. Stephen A. Spector of the University of California, San Diego, and propagated in PBMCs. Viral stocks were titered on PM1 cells. Vaccinia recombinants and PM1 cells were obtained from the NIH AIDS Research and Reference Reagent Program.

Ribozyme sequences

The following ribozyme sequences were used:

R14 5′ uuggacuuagaacuugaccagagaaacacacggacuucgguccgugguauauuaccuggua;
R392 5′ acagcaugagaaacagaccagagaaacacacggacuucgguccgugguauauuaccuggua;
R897 5′ uucuccccagaaaaggaccagagaaacacacggacuucgguccgugguauauuaccuggua.

Disabled ribozymes (Ojwang *et al.,* 1992) are altered at positions 24–26 by changing the AAA in the catalytic core to CGU.

In vitro cleavage reactions

The CCR5 ribozymes were generated by *in vitro* transcription of the synthesized DNA templates of ribozymes with T7 RNA polymerase (Welch *et al.,* 1997). The 19-nucleotide substrates were synthesized and labeled with [α-^{32}P]UTP. For the *in vitro* cleavage reaction, the concentrations of ribozymes and substrates were 0.0052 and 0.072 μM, respectively. The reactions were assembled on ice in a buffer containing 40 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 2 mM spermidine. The final volume was 25 μl. Reactions were incubated at 37°C. Then 5 μl of reaction solution was

removed and mixed with 5 μl of termination solution (10 M urea, 0.25% xylene cyanol, 0.25% bromphenol blue) at 0, 30, 60, 90, and 120 min. The reaction products were resolved on a denaturing 20% polyacrylamide gel containing 7 M urea. The degree of substrate cleavage was determined by densitometry on a Molecular Dynamics Phosphorimager (Molecular Dynamics). The kinetic properties of the ribozymes were further characterized, as described (Hampel and Tritz, 1989).

Generation of rAAV vectors and CCR5 ribozyme-expressing PM1 cells

To construct rAAV vectors, the retroviral vector, pLNL-6 (Yu *et al.,* 1993), was digested with *Sac*II, which left the 5′ LTR intact and truncated the 3′ LTR. This fragment was cloned into pSUB201 (Samulski *et al.,* 1987), a kind gift from R. J. Samulski, using *Xba*I linkers. Since the truncation of the 3′ LTR resulted in removal of the retroviral polyadenylation signal, a SV40 poly A site was added at the *Xho*I site. A tRNA ribozyme expression cassette was removed from plasmid JT-HR (Yu *et al.,* 1993) by *Eco*RI, *Sph*I digestion and blunt end-cloned into the *Hin*dIII site of the pSUB201-derived plasmid, resulting in the pAMFT plasmid. The DNA fragments encoding individual CCR5 ribozymes were generated from oligonucleotides and cloned into the *Bam*HI, *Mlu*I-digested pAMFT (Fig. 1). rAAV was generated from pAMFT as previously described (Mamounas *et al.,* 1995). PM1 cells were infected with the rAAV at an m.o.i. of 0.1. Transduced cultures were selected and expanded in RPMI medium containing 1000 μg/ml G418 for at least 3 weeks to insure that stable integration of rAAV had occurred.

Fluorescence-activated cell sorter (FACS) analysis

Cell surface CCR5 was determined by FACS analysis using PE-conjugated anti-human CCR5 monoclonal antibody 2D7/CCR5 (PharMingen). The preparation and antibody-staining of cells were carried out according to the manufacture. The FACS analysis was performed on a FACScan flow cytometer. Each cell population was gated versus the corresponding isotype control.

Cell fusion assays

Membrane fusion between HIV-1 env-expressing cells and CD4/coreceptor-expressing cells was measured by a reporter gene assay as described by Broder and Berger (Broder and Berger, 1995). Briefly, PM1 cells were coinfected with vaccinia recombinant vCB-3, which contains a human CD4 expression cassette, and vCB21R-lacZ, which contains the lacZ gene under a T7 promoter, to overexpress CD4 on the cell surface as well as to bring the lacZ gene into the cytoplasm. Meanwhile, HeLa cells were infected with vTF7-3 and either vCB43 or vSC60 to express T7 RNA polymerase in the cytoplasm and HIV-1 env on the cell surface. vTFT7-3 contains a T7 RNA polymerase expression cassette. vCB43 contains an HIV-1 Ba-L env expression cassette and vSC60 contains a HIV-1 IIIB env expression cassette. The PM1 cells (10^5) and HeLa cells (10^5) were mixed and incubated at 37°C for 2 h. The cells were then lysed and aliquots of the lysate were assayed for β-galactosidase activity with the substrate chlorophenol red β-D-galactopyranoside.

Viral challenge assay

PM1 cells and the stably transduced PM1 cells were infected with HIV-1 IIIB or Ba-L at an m.o.i. of 0.02 for a period of 2 h. The cells were washed and replated in RPMI with 10% fetal bovine serum. The cell cultures were refed with one-half volume of fresh medium every 2 to 3 days and were monitored for HIV production by p24 ELISA (Organon Technica).

Detection of CCR5 mRNA

mRNA was isolated from cells and purified using oligo-dT cellulose. One microgram of mRNA was separated on a 1% agarose–formaldehyde gel and transferred to a nylon membrane. It was then hybridized with CCR5-specific probes generated by random primers using CCR5 cDNA as the template. Messenger RNA was normalized using β-actin mRNA as a standard. β-Actin mRNA was detected by hybridizing with a β-actin probe and quantified using a Molecular Dynamics Phosphorimager. CCR5 and G3PDH (internal control) mRNA from PM1 cells was also analyzed by RT QC-PCR, in which mRNA was reverse transcribed and quantified by PCR amplification against competitive DNA mimic fragments (Clontech).

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