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Resistance to Human Immunodeficiency Virus Type 1 (HIV-1) Infection in Human CD4$^+$ Lymphocyte-Derived Cell Lines Conferred by Using Retroviral Vectors Expressing an HIV-1 RNA-Specific Ribozyme

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Toward gene therapy for the treatment of human immunodeficiency virus type 1 (HIV-1) infections in AIDS, Moloney murine leukemia virus-derived retroviral vectors were engineered to allow constitutive and *tat*-inducible expression of an HIV-1 5′ leader sequence-specific ribozyme (Rz1). These vectors were used to infect the human CD4$^+$ lymphocyte-derived MT$_4$ cell line. The stable MT$_4$ transformants expressing an HIV-1 RNA-specific ribozyme, under the control of the herpes simplex virus thymidine kinase (*tk*) promoter, were found to be somewhat resistant to HIV-1 infection as virus production was delayed. In cells allowing ribozyme expression under control of the simian virus 40 or cytomegalovirus promoter, the rate of HIV-1 multiplication was slightly decreased, and virus production was delayed by about 14 days. The highest level of resistance to HIV-1 infection was observed in MT$_4$ cells transformed with a vector containing a fusion *tk*-TAR (*trans* activation-responsive) promoter to allow ribozyme expression in a constitutive and *tat*-inducible manner; no HIV-1 production was observed 22 days after infection of these cells. These results indicate that retroviral vectors expressing HIV-1 RNA-specific ribozymes can be used to confer resistance to HIV-1 infection.

AIDS is caused by a retrovirus, human immunodeficiency virus type 1 (HIV-1), whose genome is composed of an RNA of positive polarity. Inhibition of HIV-1 multiplication could be achieved by interfering with various steps in the virus life cycle, including infection, reverse transcription, transcription, *trans* activation, translation, packaging, or release of virus particles (12). The development of HIV-1 RNA-specific ribozymes would be ideal for this purpose (16).

Ribozymes are small RNA molecules which allow sequence-specific endoribonucleolytic cleavage in a catalytic manner (reviewed in reference 17). The design of ribozyme used in this study was based on the hammerhead ribozyme found in satellite tobacco ringspot virus. This ribozyme contains a catalytic domain of 24 conserved nucleotides. Haseloff and Gerlach (5) have shown that in order to recognize the target RNA, this catalytic domain must be flanked at both the 5′ and 3′ ends by sequence that are at least 8 nucleotides long and that should be complementary to the region surrounding the cleavage site. Cleavage of target RNA occurs after a GUC, GUU, or GUA sequence and involves base-catalyzed hydrolysis of a 3′-5′ phosphodiester bond, resulting in the formation of two RNA molecules: one ending in a 5′ hydroxyl group and the other in a 2′-3′ cyclic phosphate group (5). The effectiveness of target RNA cleavage by a ribozyme depends on several factors including (i) structure (primary, secondary, and tertiary) of both the ribozyme and the cleavage site, (ii) size of RNAs containing both the ribozyme and the cleavage site, (iii) level of ribozyme-containing RNA and target RNA expression, (iv) optimal base pairing required to achieve site specificity and to allow cleavage to occur in a catalytic manner, and (v) cofactors required, if any, for cleavage to occur.

A ribozyme was designed against a conserved region within the 5′ leader sequence of HIV-1 RNA. This region was selected since it is present on all HIV-1 mRNAs and is crucial for viral reverse transcription, *trans* activation, and translation (reviewed in reference 3). Cleavage of HIV-1 RNAs at this site would give rise to two RNA molecules: (i) a truncated HIV-1 RNA, deriving from the 3′ side of the cleavage site, that would fail to reverse transcribe or translate, and (ii) an HIV-1 5′ leader RNA, deriving from the 5′ side of the cleavage site, that could act as a competitive inhibitor for replication, *trans* activation, and translation of HIV-1 RNAs. In addition, packaging of truncated HIV-1 RNA would give rise to replication-defective virus particles, which could compete with HIV-1 for receptor binding, thus preventing new rounds of infection.

The HIV-1 5′ leader sequence contains a 5′ ACUCUGGU AACUAGAGA 3′ sequence, which is conserved among various HIV-1 isolates (including NL4-3, HXB2, BRU, JH31, SF2, CDC451, RF, OYI, MAL, ELI, Z2Z6, and NDK). The HIV-1 5′ leader sequence-specific ribozyme was designed to recognize this sequence and to cleave HIV-1 RNA after the conserved GUA sequence (nucleotides 131 to 133; strain NL4-3 [1]). In this ribozyme, 8-nucleotide-long sequences complementary to either side of the cleavage site were used to confer cleavage specificity:

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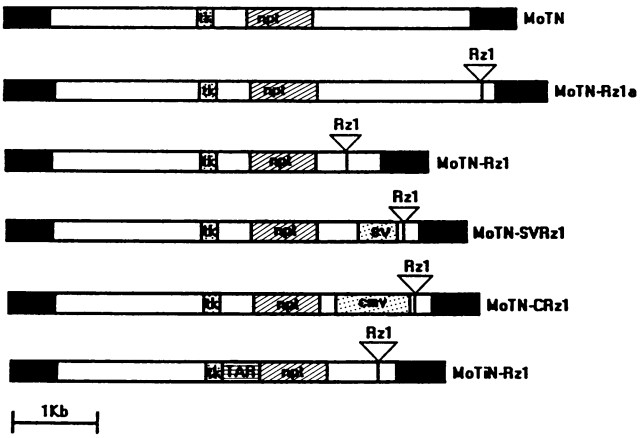

FIG. 1. Retroviral vector constructs expressing an HIV-1 RNA-specific ribozyme: MoTN, MoTN-Rz1a, MoTN-Rz1, MoTN-SVRz1, MoTN-CRz1, and MoTiN-Rz1. sv, simian virus 40; cmv, cytomegalovirus.

HIV-1 RNA 5′ . . . ACUCUGGAU↓ACUAGAGA . . . 3′
Rz1 3′ . . . UGAGACCA . . . UGAUCUCU . . . 5′

The ribozyme was expressed with a Moloney murine leukemia virus (MoMuLV)-derived retroviral vector, MoTN (8). This vector contains the MoMuLV 5′ long terminal repeat (LTR) promoter allowing retroviral vector RNA synthesis, and the herpes simplex virus (HSV) thymidine kinase (*tk*) promoter allowing neomycin phosphotransferase (*npt*) mRNA synthesis (Fig. 1). The ribozyme was expressed as part of the 3′ untranslated region of the *npt* mRNA. In case the size of ribozyme-containing RNA affected cleavage, we used internal promoters (simian virus 40 [SV40] or cytomegalovirus) to express ribozymes as part of shorter RNA transcripts. Furthermore, since *trans* activation of HIV-1 gene expression by the viral *tat* protein leads to a substantial increase in HIV-1 RNA level (reviewed in reference 14), it was anticipated that *tat*-inducible expression of ribozymes would be essential for this approach to be more effective. Thus, ribozymes were also expressed in a constitutive and *tat*-inducible manner under the control of a fusion *tk-trans* activation-responsive (TAR) promoter. The ability of these retroviral vectors expressing the HIV-1 RNA-specific ribozyme to confer resistance to HIV-1 infection was tested in human $CD4^+$ lymphocyte-derived MT_4 cell lines.

The HIV-1 5′ leader sequence-specific ribozyme was cloned as follows. All molecular biology-related techniques were performed as described in reference 9. Double-stranded deoxyribonucleotides containing ribozyme sequences flanked by 8 bp complementary to either side of the cleavage site within the HIV-1 RNA were chemically synthesized (Queen's University, Kingston, Ontario, Canada). These oligonucleotides were flanked by a *Bam*HI site at the 5′ end and by *Cla*I and *Sph*I sites at the 3′ end. Their nucleotide sequences were as follows:

$Rz1^+$: 5′$_p$gatccTCTCTAGTCTGATGAGTCCGTGAGGACGAAACCAGAGTatcgatagcatg

$Rz1^-$: 5′$_p$ctatcgatACTCTGGTTTCGTCCTCACGGACTCATCAGACTAGAGAg

Sequences represented with lowercase letters correspond to restriction sites, and those underlined correspond to HIV-1 sequences flanking the catalytic domain of the ribozyme.

The vectors pBRMoTN-Rz1a and pBRMoTN-Rz1 were engineered to allow ribozyme expression under control of the MoMuLV 5′ LTR and HSV *tk* promoters (Fig. 1). In these vectors, ribozymes were expressed as part of the 3′ untranslated region of the *npt* mRNA. The double-stranded ($Rz1^+$ and $Rz1^-$) oligonucleotides were cloned in pBR322 at the *Bam*HI-to-*Sph*I sites. The *Cla*I-*Cla*I (~500-bp) fragment containing pBR322 sequences from *Cla*I to *Bam*HI and ribozyme sequences from *Bam*HI to *Cla*I was then cloned into the pBRMoTN vector at the *Cla*I site. Clones containing ribozyme sequences in the correct orientation were selected by restriction enzyme analysis. The resulting vector was called pBRMoTN-Rz1a. From this vector, the pBRMoTN sequences present within the 3′ untranslated region of *npt* mRNA (from *Bam*HI to *Cla*I) and the pBR322 sequences cloned together with the ribozyme (from *Cla*I to *Bam*HI) were removed by *Bam*HI deletion. The resulting vector was called pBRMoTN-Rz1. In these and subsequent vectors, the presence of the ribozyme sequence was confirmed by slot-blot analysis with a synthetic DNA probe containing 17 nucleotides corresponding to the catalytic domain of the ribozyme.

The vectors pBRMoTN-SVRz1 and pBRMoTN-CRz1 were engineered to allow ribozyme expression under control of the MoMuLV 5′ LTR, HSV *tk*, and SV40 or cytomegalovirus promoters (Fig. 1). The pBRMoTN-SVRz1 vector was constructed by cloning the *Bam*HI-to-*Bgl*II fragment of pMSG-CAT (containing the SV40 promoter sequences; Pharmacia) at the *Bam*HI site in pBRMoTN-Rz1. Clones containing the SV40 promoter in the correct orientation were selected by restriction enzyme analysis. The pBRMoTN-CRz1 vector was constructed in the following manner. The *Bgl*II-to-*Bam*HI fragment of pBRMoTN-Rz1 was replaced by the *Bgl*II-to-*Bam*HI fragment from CDM-nef, providing part of the *npt* gene as well as the cytomegalovirus promoter. The correct clones were selected for the presence of these restriction sites.

The vector pUCMoTiN-Rz1 was engineered to allow ribozyme expression under control of the MoMuLV 5′ LTR and *tk*-TAR fusion promoters (Fig. 1). The *tk*-TAR promoter was constructed (5a) by fusing the HSV *tk* promoter to the HIV-1 *tat* mRNA leader sequence (nucleotides +1 to AUG); this region contains the TAR element required for *trans* activation by the *tat* protein (13–15). This promoter is, therefore, expected to allow constitutive and *tat*-inducible gene expression. To construct pUCMoTiN-Rz1, ribozyme sequences were first cloned into pUCMoTN by replacing the *Xho*I-*Cla*I fragment of pUCMoTN by the ribozyme-containing *Xho*I-*Cla*I fragment from pBRMoTN-Rz1; the resulting vector was called pUCMoTN-Rz1. The *Xho*I-*Hin*dIII fragment of pUCMoTN-Rz1 was then replaced by an *Xho*I-*Hin*dIII fragment containing the *tk*-TAR sequence and lacking the ribozyme cleavage site from pUCMoTiN vector previously constructed in our laboratory (5a).

To generate retroviral vector particles, we used the retroviral vectors pBRMoTN, pBRMoTN-Rz1a, pBRMoTN-Rz1, pBRMoTN-SVRz1, pBRMoTN-CRz1, and pUCMoTiN-Rz1 to transfect the ecotropic packaging cell line Psi-2 (10) as described in references 4, 6, and 18. Vector particles released from the transformed Psi-2 cells were then used to infect the amphotropic PA317 cell line (11) as described in reference 7. The transformed PA317 cells releasing retroviral vector particles MoTN, MoTN-Rz1a, MoTN-Rz1, MoTN-SVRz1, MoTN-CRz1, and MoTiN-Rz1 were then used to infect the human $CD4^+$ lymphocyte-derived MT_4 suspension cells by cocultivation. Essentially, 10^6 MT_4 cells in 10 ml of RPMI 1640 medium containing L-glutamine (2 mM), antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml;

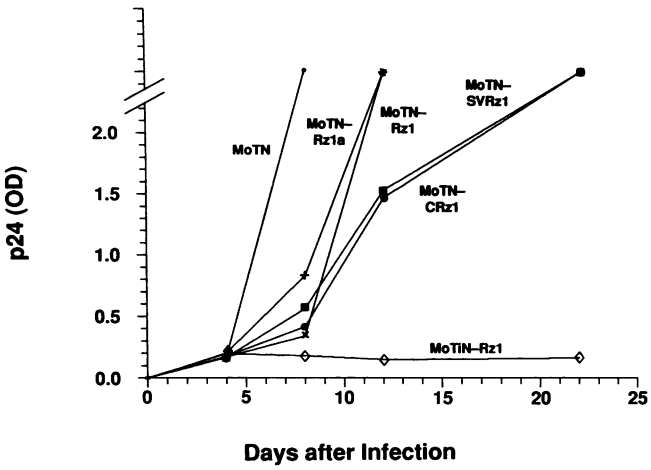

FIG. 2. Susceptibility of MT_4 cells expressing an HIV-1 RNA-specific ribozyme to infection by HIV-1_{NL4-3}. MT_4 cells transformed with MoTN, MoTN-Rz1a, MoTN-Rz1, MoTN-SVRz1, MoTN-CRz1, or MoTiN-Rz1 were infected with HIV-1_{NL4-3}, and the level of p24 antigen released in the culture supernatant was determined by ELISA at various times after infection. (1 OD unit = 200 pg of HIV-1). The maximum OD value reported is 2, since values greater than this could not be accurately measured because of the sensitivity of the ELISA reader. Values of >2 are shown at an arbitrarily chosen point.

amphotericin B [Fungizone], 0.25 μg/ml; GIBCO), and fetal calf serum (10%; GIBCO) were placed on 100-mm tissue culture dishes containing transformed PA317 cells which had reached 50% confluency. After 24 h of cocultivation at 37°C, the MT_4 cells in suspension were transferred to 100-mm petri dishes and were cultured in RPMI 1640 medium supplemented as above and containing G418 (400 μg/ml; GIBCO). The G418-resistant stable MT_4 transformants were selected for by growth in G418 medium for up to 3 to 4 weeks (time required for untransformed cells to die). The stably transformed G418-resistant cells (without any cloning) were then used for RNA analysis and subjected to HIV-1 challenge.

MT_4 cells transformed with MoTN, MoTN-Rz1a, MoTN-Rz1, MoTN-SVRz1, MoTN-CRz1, or MoTiN-Rz1 were challenged with HIV-1. Virus produced from a cloned HIV-1 provirus, strain NL4-3, was obtained from the National Institutes of Health and was used without further propagation in our laboratory. First, virus titer was determined for infection of MT_4 cells (2). A 20-μl portion of virus was then used to infect 2×10^6 MT_4 transformants in 2 ml of medium; infections were done in duplicate and were repeated twice. HIV-1 production was monitored at various time intervals by measuring the HIV-1 p24 antigen (*gag* gene product) levels in the culture supernatant by enzyme-linked immunosorbent assay (ELISA), using a kit purchased from Abbott. These results are shown in Fig. 2. The control consisted of MT_4 cells transformed with MoTN, which lacked the ribozyme sequence. Eight days postinfection, the p24 antigen level in the culture supernatant from control cells reached optical density (OD) values of >2.0. In contrast, the p24 antigen levels in the culture supernatants from MT_4 cells containing MoTN-Rz1a and MoTN-Rz1 required 12 days to reach OD values of >2.0. Thus, MoTN-Rz1a and MoTN-Rz1 vectors were able to make MT_4 cells somewhat resistant to HIV-1 infection as virus production was delayed by about 4 days compared with the control (Fig. 2). The MT_4 cells expressing MoTN-SVRz1 and MoTN-CRz1 seemed to be more resistant to HIV-1 infection as the rate of virus production was decreased and the time required for p24 antigen levels in the culture supernatant to reach OD values of >2.0 was delayed by about 14 days compared with the control (Fig. 2). The MT_4 cells expressing MoTiN-Rz1 were the most resistant as no virus could be detected in their culture supernatant for up to 22 days after infection (Fig. 2).

The approximate sizes of these RNAs expressed from the MoMuLV 5′ LTR, HSV *tk* or *tk*-TAR, and SV40 or cytomegalovirus promoter are expected to be 4.5, 2, and 0.5 kb, respectively. As revealed from Northern (RNA) blot analysis, similar levels of 4.5- and 2-kb RNAs are expressed in various MT_4 transformants in the absence of *tat* protein; the presence of 0.5-kb RNA could not be detected (data not shown).

The various levels of resistance observed in MoTN-Rz1 and MoTN-SVRz1 or MoTN-CRz1 are probably due to the size of ribozyme-containing RNAs expressed in the MT_4 transformants. The *npt* mRNA expressed from the *tk*-TAR promoter in MoTiN-Rz1 contains TAR sequence at the 5′ end and the ribozyme sequence as part of the 3′ untranslated region. Increased HIV-1 resistance observed in MT_4 cells expressing MoTiN-Rz1 compared with MoTN-Rz1 could be due to a combination of *tat*-inducible gene expression, the presence of the HIV-1 TAR sequence, and the presence of the ribozyme sequence.

Cell counts were performed on day 22 after HIV-1 infection. The MT_4 transformants containing any of the five ribozyme-expressing vectors gave a cell count between ~0.1 $\times 10^6$ and 0.3×10^6 cells per ml. The control MT_4 transformants containing MoTN gave a cell count of 0.02×10^6 cells per ml. From these results, it seems that although virus production is evident from the p24 antigen levels in the culture supernatants from MT_4 cells containing MoTN-Rz1a, MoTN-Rz1, MoTN-SVRz1, MoTN-CRz1, and MoTiN-Rz1, these transformants are somewhat resistant to the lethal effect of HIV-1 infection compared with the control. The underlying mechanism for this resistance remains to be determined.

In conclusion, a retroviral vector expressing an HIV-1 5′ leader sequence-specific ribozyme in a constitutive and *tat*-inducible manner was shown to confer resistance to HIV-1 infection in human $CD4^+$ lymphocyte-derived MT_4 cell lines. This vector containing the *tk*-TAR fusion promoter will be useful for expressing other anti-HIV-1 genes as well. The precise step(s) within the HIV-1 life cycle that is blocked by the HIV-1 5′ leader sequence-specific ribozyme remains to be determined. The MoTiN-Rz1 vector developed in this study will now be used to transform human bone marrow stem cells, and the ability of transformed human stem cells to give rise to an HIV-1-resistant immune system upon transplantation and differentiation in animal model systems will be determined.

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