Inhibition of infection of incoming HIV-1 virus by RNA-cleaving DNA enzyme

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Abstract Nine different DNA enzymes (DzV3-*n*, *n* = 1–9) targeting the V3 loop region of HIV-1 HXB2 were synthesized. One of those, DzV3-9, efficiently cleaved the target in the conserved sequence in the RNA transcript in vitro. DzV3-9 was stable in the cells and inhibited replication of both NL432 and SF162 strains in U87 cells expressing CD4 and co-receptors. The inhibitory effect of DNAzyme on incoming HIV-1 was also demonstrated with pseudotype virions generated by NL432-based luciferase reporter genes. Thus, an efficient, stable DNAzyme against a functionally important region of HIV-1 was identified, and it may be useful for prevention of HIV-1 infection.

Key words: DNA enzyme; Inhibition of human immunodeficiency virus type 1 infection; V3 loop; Gene therapy

1. Introduction

DNA in biological systems has long been considered a passive molecule carrying genetic information. Single-stranded DNA can also fold into well-defined, sequence-dependent tertiary structures, specifically bind to a variety of target molecules, and exhibit catalytic activities similar to those of ribozymes or protein enzymes [1,2]. Catalytic DNA enzymes (DNAzymes) have been generated using in vitro selection methodology. DNAzymes have been shown to target single-stranded DNA or RNA, and to catalyze chemical reactions such as phosphoester transfer, phosphoester formation, porphyrin metalation, and RNA or DNA cleavage in the presence of divalent cations [3–6]. Santoro and Joyce generated an RNA-cleaving DNAzyme which cleaved synthetic oligo-RNAs corresponding to the start codon regions of HIV-1 gag/pol, env, vpr, tat, and nef [7]. DNAzymes are easier to prepare and less sensitive to chemical and enzymatic degradation than ribozymes. However, the application of DNAzymes in biological contexts, for example as antiviral agents, has not yet been reported.

Acquired immune deficiency syndrome (AIDS) is etiologically associated with human immunodeficiency virus (HIV) infection. Infectious virions of HIV contain two identical copies of single-stranded RNA. The replication cycle of HIV is divided into early and late phases. The early phase begins with attachment of a virion to a cell surface receptor followed by integration into the host cell genome. The late phase begins with transcription and processing of viral RNA from the integrated proviral template and ends with release of progeny virions from the cell. A recent investigation demonstrated high plasma HIV RNA levels in infected individuals, and their prognostic importance [8]. Cleaving the genome RNA of incoming virus prior to integration in the early phase as well as the new transcribed viral RNA in the late phase would effectively interrupt the viral replication cycle. Envelope glycoproteins (Env) of HIV-1 play a major role in infection. The V3 loop in Env is formed by a disulfide bridge between invariant cysteines at positions 303 and 338 of HIV-1 HXB2 Env protein (GenBank accession number K03445). Although the V3 loop resides in a variable region, both of its flanking sequences, including the two invariant cysteine residues, and a central tip sequence are highly conserved [9,10]. Previously, we synthesized phosphorothioate DNA/RNA chimeric ribozymes against seven different regions within the V3 loop region of HXB2. One of them (RzV3-1T), targeted against the conserved sequences, could specifically suppress the replications of both T-cell- (X4) and macrophage-tropic (R5) viruses by reducing envelope gene expression [11].

Our original aim in this study was to clarify if DNAzymes could inhibit infection by HIV-1 virus. We therefore synthesized RNA-cleaving DNAzymes against nine different sequences within the V3 loop in HIV-1 Env. Based on in vitro cleavage reactions, the most efficient DNAzyme was selected, and its intracellular stability was studied using FITC-labeled DNAzyme. The effect of DNAzymes on infection by incoming HIV-1 virus was also demonstrated.

2. Materials and methods

2.1. Design and synthesis of DNAzymes

Based on Santoro and Joyce's report, we identified nine different target sequences for RNA-cleaving DNAzymes, each of which included an AU in the V3 loop region of HXB2 cDNA between the two invariant cysteines (7110–7217). The corresponding DNAzymes (DzV3-*n*) and their inactive controls (DzV3-*n*C, T15G) (*n* = 1–9) were chemically synthesized (Fig. 1A,B). The fluorescent isothiocyanate (FITC)-labeled DNAzymes were made by introducing FluoroPrime at the 5′ end. Random oligo-DNA (GTCAGTCAGTCAGTCAGTCA) was also synthesized. All the above compounds were synthesized by Takara Co., Kyoto, Japan.

2.2. Plasmid constructions

A plasmid expressing the *env* gene of HXB2, pSVIIIenv3-2 [12], was kindly provided by Dr. J. Sodroski, and was used to generate V3 loop RNA transcripts for in vitro cleavage assays. For generation of luciferase reporter gene-containing pseudotype virus, envelope genes of two T-cell-tropic strains, HXB2 and SF2, and two macrophage-tropic strains, SF162 and ADA [13], subcloned in pSM vector were used. pSMHXB2 [14] and pSMADA were kindly provided by Dr. C. Weiss. pSMSF2 and pSMSF162 were kindly provided by Dr. A. Koito [15]. *P. pyralis* luciferase expression vector pNL4-3-Luc-E$^-$R$^-$, which con-

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tains a frameshift mutation near the 5′ end of *env* in the *Nde*I site of NL43, was kindly provided by Dr. D. Littman [16].

2.3. Cell culture

All cells were cultured at 37°C in 100% humidified air containing 5% CO_2. A human embryonic kidney cell line, 293T [17], and human astroglia cell lines expressing CD4 antigen and coreceptor CXCR4 (cell line U87.CD4.CXCR4) or CCR5 (cell line U87.CD4.CCR5) were kindly provided by Dr. D. Littman [18], and were used as the target cells for T-cell- and macrophage-tropic viruses, respectively. The cells were grown in Dulbecco's modified Eagle's medium containing high glucose (Gibco BRL, Grand Island, NY, USA) and 10% fetal calf serum (FCS). 300 μg/ml G418 (geneticin disulfate, Wako Pure Chem., Osaka, Japan) and 1 μg/ml puromycin (Sigma, St. Louis, MO, USA) were added as selection reagents for U87.CD4 with coreceptors (D-MEM complete medium). The viability of the cells was estimated using the trypan blue dye exclusion method.

2.4. In vitro cleavage assay

A DNA fragment from the V3 region in pSVIIIenv3-2 was amplified by PCR using oligonucleotide primers as described previously [10]. The product was subcloned into pT7Blue T-vector, and used as a template for in vitro RNA transcription with T7 RNA polymerase. The RNA transcript was labeled with [α-^{32}P]UTP during the transcription and the concentration of the transcript was measured with a DNA Dipstick kit (Invitrogen, San Diego, CA, USA). The quantified transcript was subjected to in vitro cleavage by DNAzymes. Concentrations of both substrates and DNAzymes ranged from 2 nM to 20 μM. Autoradiograms of gels were analyzed with a Bio-Image analyzer (BAS2000) and cleavage products were quantitated using a densitometer (PDI system, Huntington Station, NY, USA) as described previously [19]. Time points were selected to represent the linear burst and steady-state velocity phases of the catalyst-substrate pair being tested. Values of apparent K_m and K_{cat} were calculated as described [11].

2.5. Detection of DNAzymes in the cells

To observe the stability and localization of DNAzymes, 2×10^4 cells were seeded in each well of 8-well Lab-Tek chamber slides (Nunc, Naperville, IL, USA) 1 day before the experiment, and the FITC-labeled DNAzymes were delivered into U87.CD4.CXCR4 cells using lipofectamine with Opti-MEM (Gibco BRL) according to the manufacturer's instructions. After 2 h of exposure, the cells were rinsed twice with D-MEM complete medium and then cultivated in the same medium. At the indicated times, cells on the slides were subjected to confocal fluorescence microscopy (Axiophot, Zeiss, Jena, Germany) using a Bio-Rad MRC 600 laser confocal module (Bio-Rad Lab, Hercules, CA, USA).

2.6. Virus production

An X4 strain, NL432, and two R5 strains, SF162 and ADA, were used for infection. The target sequences in the V3 loop region of NL432 and SF162 are identical with that of HXB2, while that of ADA has a single base substitution near the cleavage site (Table 1). NL432 was produced by transfection with the proviral DNA clone pNL432 [20] into 293T cells. SF162 and ADA were propagated in peripheral blood mononuclear cells activated by 10 μg/ml phytohemagglutinin (Difco Lab., Detroit, MI, USA) as described [15]. The supernatants were clarified by centrifugation followed by filtration, and the levels of p24, the core antigen of HIV-1, were determined by the antigen capture assay (Abbott Laboratories, Abbott Park, IL, USA). The amounts of virus were expressed as the concentrations of p24 antigen.

2.7. Infection

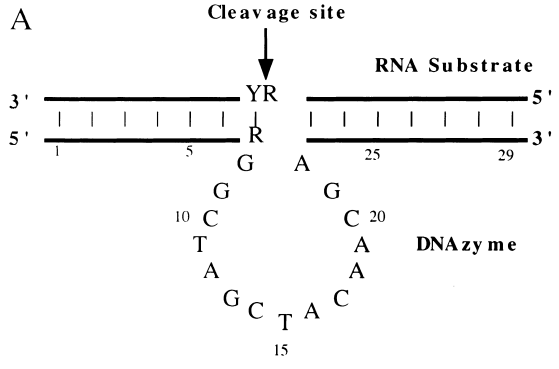
U87.CD4.CXCR4 or U87.CD4.CCR5 cells were used as target cells for X4 or R5 virus infection, respectively. One day before infection, 2×10^4 target cells were seeded per well in 48-well plates. Two hours before infection, DNAzymes (8, 16, 32, 64, or 128 pmol) were introduced into the target cells using lipofectamine as described above. After removal of the extracellular DNAzymes, the cells were gently washed twice with D-MEM complete medium and then infected with virus (5 ng or 10 ng of p24 antigen) for 6 h in the presence of 8 μg/ml polybrene. The cells were then cultured after they were washed twice with the same medium. The supernatants were obtained at the indicated times and subjected to the antigen capture assay. The amount of p24 antigen produced by infected cells not treated with DNAzymes was used as a control to calculate the percent inhibition by DNAzymes.

2.8. Production of luciferase receptor gene-containing pseudotype virus and luciferase assay

The effects of DNAzymes on the replication of incoming virus were examined by using pseudotype viruses containing luciferase reporter gene, pNL43-Luc-E-R-. To confirm the specific activity of DNAzyme on target sequence, the V3 loop corresponding region in pNL43-Luc-E-R- was deleted by removing the *Bgl*II-*Bgl*II fragment (7031–7611), and the resultant plasmid was named pNL4-3-Luc-V3$^-$, which was used as control [11]. These pseudotype viruses were obtained 36 h after co-transfection of luciferase reporter gene-containing vectors, and one of the Env-expressing vectors (pSMSF162, pSMADA, pSMSF2 or pSMHXB2) into 293T cells. Pseudotype virus (10 ng of p24 antigen/well) was used to infect target cells seeded in 48-well plates (2×10^4 cells/well). The infected cells were pretreated with DNAzymes (64 pmol/well) for 2 h and were harvested at 36 h post inoculation, and the luciferase activity of each lysate was measured with a luminometer (Bio Orbit 1251 luminometer, Turku, Finland). The luciferase activity in cells not treated with DNAzymes was used as a control to calculate the inhibition by the DNAzymes.

3. Results and discussion

To test the possible application of DNAzymes against infection by incoming HIV-1 virus, we selected target sequences within the V3 loop region of HIV-1. This region was selected because HIV infection was reported to be inhibited by ribozymes targeted against the conserved sequences of the V3 loop region [11,19].



B 7094-ucuguagaaAU[1]uaauuguacaagacccaa caacaAU[2]acaagaaaaagaAU[3]ccguAU[4]cca gagaggaccagggagagcAU[5]uuguuacaAU[6]ag gaaaaAU[7]aggaaauAU[8]gagacaagcacAU[9]u guaacauu-7222

Fig. 1. Structures of DNAzymes and their target sequences. A: A substrate and a DNAzyme. The flanking sequence of the DNAzyme (bottom strand) binds to the substrate (top strand) through Watson-Crick pairing. Cleavage occurs at the position indicated by the arrow. R = A or G; Y = U or C. Sequences of the inactive control DNAzymes were the same as their corresponding wild-type DNAzymes except for the substitution of G13 in the cleavage core by A13. B: The target sequence in the HXB2 V3 loop region (7094–7222) is shown. Individual target sequences, expressed by bold capital characters, are numbered.

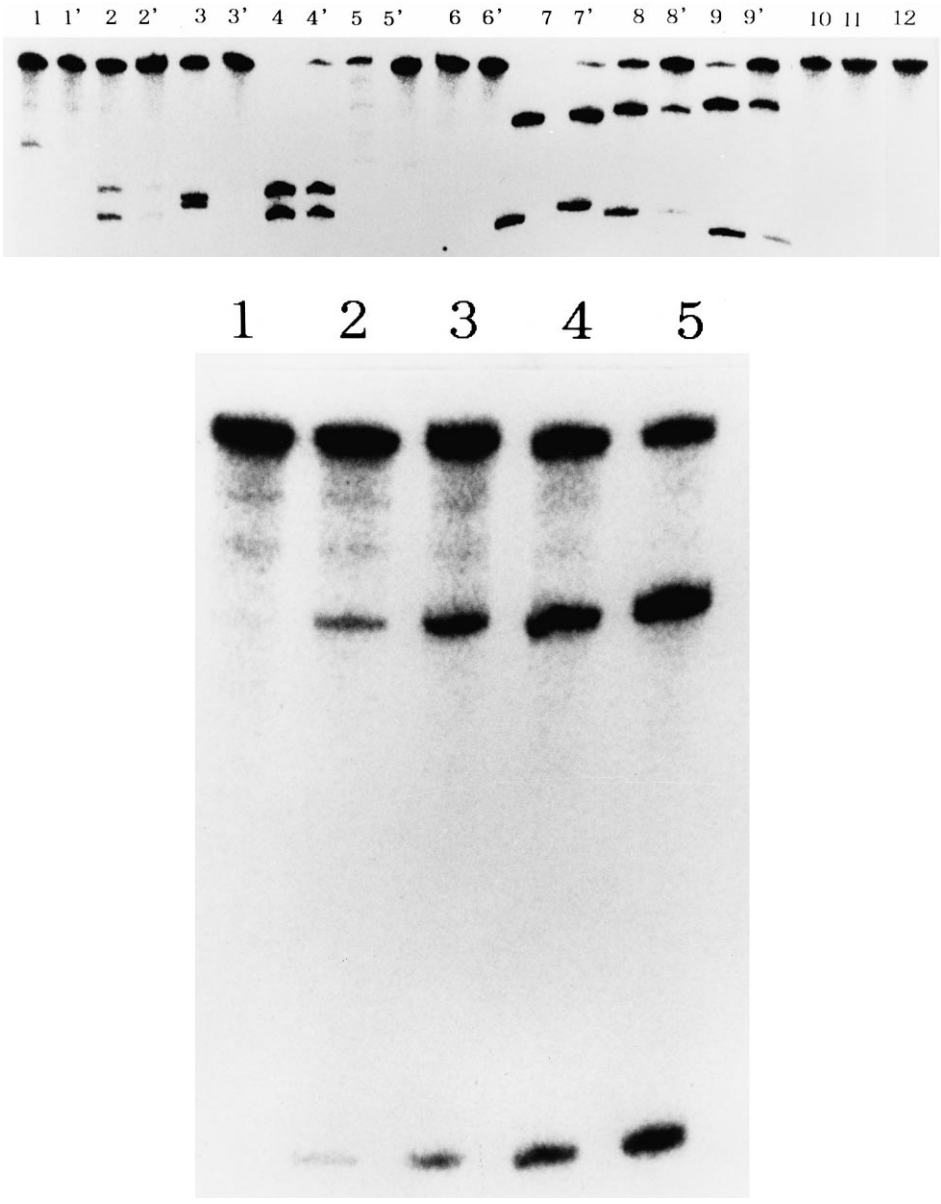


Fig. 2. In vitro cleavage by DNAzymes. A: Cleavage of HXB2-RNA by nine different DNAzymes. The reactions were performed at 37°C for 1 h. The molar ratio of DNAzymes to substrates was 100:1. The most slowly migrating band is the intact RNA transcript, and the faster migrating bands are the cleavage products. The numbers of the lanes from 1 to 9′ correspond to DNAzymes from DzV3-1 to DzV3-9, respectively and the prime symbol indicates the substitution T15G in the DNAzyme. Lane 10 contained mutated DzV3-9 (G13A), which was used as inactive control in the following experiment. Lane 11 contained the antisense from DzV3-9 with substrate RNA. Lane 12 contained intact RNA transcript only. B: Time-dependent cleavage of HXB2-RNA by DzV3-9. Representative experiments with a ratio of 100:1 of DzV3-9 to substrate are shown. The cleavage products (277 and 137 bases) of HXB2-RNA (414 bases) were as expected. The lane numbers indicate the following reaction times: 1: 1 min; 2: 5 min; 3: 10 min; 4: 20 min; 5: 40 min.

3.1. In vitro cleavage reaction and kinetic analysis

The amounts of cleavage catalyzed by DzV3-*n* were examined using the V3 RNA transcript of HXB2 (HXB2-RNA) as substrate (Fig. 2A). After 2 h of incubation, more than 50% of the substrate was cleaved by DzV3-4, -7, -8, and -9. However, little or no cleavage was seen when other DNAzymes were used. The reason why these four DNAzymes showed higher cleavage activities than other DNAzymes remains to be elucidated. The tertiary structure of the target RNA probably affects the results, because all of the target nucleotides were AU. The target sequences for DzV3-4, -7, and -8 are located in the variable region, while that for DzV3-9 is located in the

Table 1
Target sequences for DzV3-9 in the V3 loop region of various HIV-1 strains

Strain	Target sequence	Co-receptor usage
HXB2[a]	CAAGCACATTGTAAC	X4
NL432	———————	X4
SF2	A———————	X4/R5
SF162	———————	R5
ADA	——————C—	R5

[a]7206–7220, according to GenBank accession number K03455.

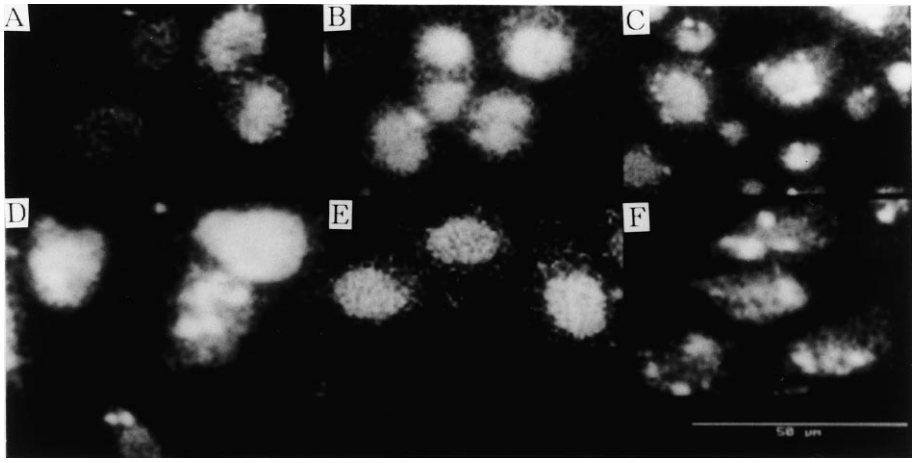


Fig. 3. The stability of DNAzymes in the cells. Fluorescent-labeled DNAzyme(DzV3-9) was delivered into U87.CD4.CXCR4 cells. The cells were subjected to confocal fluorescence microscopy at the indicated times (A: 2 h; B: 6 h; C: 9 h; D: 12 h; E: 24 h; F: 48 h).

conserved region. Therefore, we used DzV3-9 for further investigations.

According to Santoro and Joyce, the catalytic core tip could be T, A, or C, with T giving the highest efficiency of cleavage. In our preliminary experiments, we used G at the core tip as an inactive control DzV3-nC (T15G), however, modest to moderate amounts of cleavage products were observed when it was incubated with HXB2-RNA, and the amounts of cleavage products were comparable to those produced by T15A and T15C. Therefore, we performed in vitro cleavage assays using DNAzymes with single-base random mutations within the conserved loop region from 8G to 22A of DzV3-9 (the numbering is shown in Fig. 1). Several mutated DNAzymes (G13A, A16G, C17A, A19G, C20T, and G21A) did not show any cleavage products. We arbitrarily chose G13A as control DNAzyme (DzV3-9C) in the following experiments. The 414-base RNA transcript incubated with DzV3-9 was cleaved with the expected sizes: 277 bases of the 5′ fragment and 137 bases of the 3′ fragment. The substrates incubated with DzV3-9C did not give any detectable cleaved product, indicating that the catalytic reactions induced by DzV3-9 were specific. A more detailed series of such in vitro assays could be used to analyze the structure-function relationships of the DNAzyme.

The catalytic activities of DzV3-9 against HXB2-RNA transcripts were examined and representative data are shown in Fig. 2B. The K_{cat} was 2.85×10^{-4} min^{-1}, the apparent K_m was 9.41×10^{-2} μM, and the K_{cat}/K_m was 3×10^{3} M^{-1} min^{-1}. The catalytic activities and the affinities of DzV3-9 for HXB2-RNA were reduced to 1/100 and 1/10, respectively, of those previously found when 15- or 17-nt synthetic RNA substrates were used [7]. We also compared the activities with those of a stabilized ribozyme, RzV3-1T, targeting another conserved region of the V3 loop. The same RNA transcripts were used as substrates. The K_{cat} of DzV3-9 was reduced to 1/4 that of RzV3-1T; however, the K_m was also reduced to about 1/10 that of RzV3-1T, indicating that the affinity for the substrate of DzV3-9 was higher than that of RzV3-1T. Therefore, the overall kinetic activity, expressed by K_{cat}/K_m was increased by 2-fold compared to that of RzV3-1T.

3.2. The stability of DNAzymes in the cells

Fluorescent signals in the cells were observed after introduction of labeled DNAzymes. Two hours after introduction, fluorescent signals were detected in both cytoplasm and nucleus in cells treated with DzV3-9 (Fig. 3). Essentially similar signals were observed up to 24 h. At 48 h, the signals became weak, but were still detectable in both nucleus and cytoplasm. In our previous work, the intracellular signal by wild-type ribozyme (all RNA) faded within 6 h after introduction, whereas that of stabilized ribozyme, which contained 80%

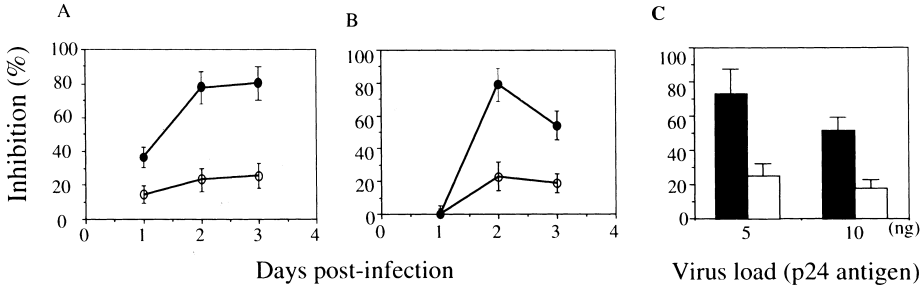


Fig. 4. Effect of DzV3-9 on virus replication. A and B: U87.CD4.CXCR4 cells infected with NL432 virus (A: 5 ng; B: 10 ng of p24 antigen). The *y*-axis shows the percent inhibition of p24 antigen production from DzV3-9-treated target cells. The production of p24 in control cells is (A) 0.1 ng, 8 ng and 180 ng, and (B) 0.2 ng, 15 ng, and 298 ng on days 1, 2 and 3, respectively. The *x*-axis shows the time of harvest of supernatants. ●: DzV3-9 treatment; ○: DzV3-9C treatment. C: U87.CD4.CCR5 cells infected with SF162 strain on day 3. The *y*-axis shows the percent inhibition of p24 antigen production in DzV3-9-treated target cells. The production of p24 in control cells of 5 ng or 10 ng infection with SF162 is 160 ng or 258 ng, respectively. The *x*-axis shows the load of infecting virus. ■: DzV3-9 treatment; □: DzV3-9C treatment. The means and standard deviations from three independent experiments are shown.

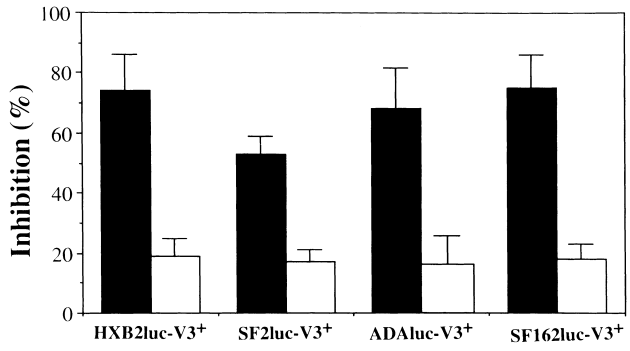


Fig. 5. Effect of DzV3-9 on the infection with incoming virus. The *y*-axis shows percent inhibition of luciferase activity. The luciferase activity in control cells is 2.69×10^4, 1.54×10^4, 2.71×10^4, or 2.4×10^4, respectively. The means and standard deviations from three independent experiments are shown. The *x*-axis shows different types of incoming virus. DzV3-9-treated cultures showed significantly greater inhibition than DzV3-9C-treated cultures. ■: DzV3-9; □: DzV3-9C.

phosphorothioate DNA linkages, was still detectable even after 24 h. Therefore, the stability of DNAzyme in the cells was similar to the phosphorothioate DNA/RNA chimeric ribozymes.

3.3. DNAzyme inhibited HIV-1 virus replication

The effect of DzV3-9 on virus replication was determined and was expressed as percent inhibition. The viability of DNAzyme-treated U87 cells was greater than 95% before infection, and apparent degenerative changes in the cells were not seen by microscopic observation after DNAzyme treatment followed by virus infection.

The inhibitory activity of DzV3-9 on NL432 replication was dose-dependent in U87.CD4.CXCR4 cells, and the optimal inhibition was seen at 64 pmol/well (data not shown), while less ribozyme (16 pmol) was required in the similar assay [19]. Therefore, we used 64 pmol of DzV3-9 to treat the target cells before infection in the following experiments. Two different dosages (5 ng or 10 ng of p24 antigen) of virus were used for infection. Treatment with DzV3-9 suppressed the virus replication by 77% (5 ng) or 79% (10 ng) on day 2, and 80.7% (5 ng) or 54% (10 ng) on day 3 (Fig. 4A,B). The inhibitory activity of DzV3-9 on ADA and SF162 virus replication was also tested in U87.CD4.CCR5 cells. The maximal suppression of SF162 virus replication was observed on day 3, with 68% and 48% suppression for 5 and 10 ng of infecting p24 antigen, respectively (Fig. 4C). DzV3-9C also caused moderate (less than 30%) inhibition of virus replication, however random oligo-DNA did not cause any inhibition (data not shown). The catalytic loop in DzV3-9C was inactivated while the target-binding fragment was intact. Therefore, the moderate inhibition by DzV3-9C could be explained by its antisense effect. Moreover, DzV3-9 caused little inhibition of ADA virus replication (data not shown). The lack of a suppressive effect on the ADA strain might be explained by a single base substitution near the DNAzyme cleavage site. Comparable inhibitory activity was achieved at a higher concentration of DzV3-9 than that of RzV3-1T. Furthermore, it is known that many factors are involved in determining the activity of ribozymes in vivo, such as RNA-binding proteins and co-localization of substrate and ribozyme [21]. Similar factors, and also DNA-binding proteins may affect the activity of DNAzymes in vivo. The identification of such cellular factors and their interactions with DNAzymes should be elucidated.

3.4. DNAzyme inhibited the infection of incoming virus by single-cycle infection assay

The HIV-1 replication cycle is divided into the early and late phases. The effect of DNAzymes on replication of incoming virus was elucidated by a sensitive single-cycle infection system using a luciferase reporter gene. The inhibition by DzV3-9 was not seen in infection with V3 loop-deleted pseudotype virus, HXB2luc-V3$^-$ [11] (data not shown). U87.CD4.CXCR4 cells were infected with the pseudotype reporter viruses coated with Env from HXB2 (HXB2luc-V3$^+$) or SF2 (SF2luc-V3$^+$), and U87.CD4.CCR5 cells were infected with the pseudotype reporter viruses coated with Env from ADA or SF162 (ADAluc-V3$^+$ or SF162luc-V3$^+$). The luciferase activity of pseudotype virus in cells treated with DzV3-9 was markedly lower than that in cells not treated with DNAzyme, the inhibition was 74% on HXB2luc-V3$^+$, 54% on SF2luc-V3$^+$, 72% on SF162luc-V3$^+$, or 74% on ADAluc-V3$^+$ (Fig. 5). These viruses all contained the same reporter gene, although they were coated with different viral Env. The lower inhibition by DzV3-9 against SF2luc-V3$^+$ could not be explained by the different co-receptor usage because the inhibition of HXB2luc-V3$^+$ infection, which also uses CXCR4, was over 70%. It could also not be ascribed to a higher infection efficiency of SF2luc-V3$^+$ because luciferase activity in SF2luc-V3$^+$-infected cells was lower than that in the other pseudotype virus infected cells (see legend to Fig. 5). It should also be noted that a complete inhibition could not be achieved by either DNAzyme or ribozyme. The reason for the resistance of some viruses remains to be elucidated.

This is the first report demonstrating the biological activity of DNAzyme. In comparison with modified ribozyme, DNAzyme was easier to synthesize, and has similar stability, though a higher concentration should be administered. The fate of DNA administered into the body has not been well characterized. However, it was reported that DNA vaccine encapsulated in microparticles could enter mucous macrophages [22], which are the initial targets for HIV infection. The primary infection of HIV is known to be mediated by R5 virus via macrophage/dendritic cells. The inhibitory effect against incoming R5 viruses (ADA, SF162) by DNAzymes implied that DNAzymes could prevent HIV infection if they were taken up by the appropriate target cells. As targets for DzV3-9, 15 nucleotide long sequences identical to that of HXB2 are present in 70% of HIV-1 clade B group (Los Alamos databases, 1997). If we could include the one single base mismatch at the distal site, the coverage of DzV3-9 would increase by 91%.

Taken together, these data suggest that DzV3-9 is a promising agent, because (1) DzV3-9, directed against the conserved sequence of the V3 loop of HIV-1 HXB2, cleaved its target RNA transcript specifically in vitro, (2) DzV3-9 inhibited infection by both X4 and R5 strains, (3) DzV3-9 inhibited infection by incoming virus.

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