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Inhibition of HIV-1 infection in cells expressing an artificial complementary peptide☆

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Abstract

TLMA2993 peptide (N′-TLMALELKGKLLLAGLAPSAFLPLSFPEGL-C′) which was designed by a computer program (MIMETIC) inhibited the activity of HIV-1 reverse transcriptase in a cell-free system. Therefore, we constructed a TLMA2993 expression vector containing an artificial cDNA for TLMA2993 to generate the peptide in cells. The cell lysate of transfected U937 cells contained a detectable level of TLMA2993 peptide using competitive ELISA. The transfectants were resistant to HIV-1 infection due to expression of TLMA2993 peptide in the cells. The use of MIMETIC to design an inhibitory peptide to any intracellular target molecule, followed by transfection of the artificial cDNA for the peptide, could afford a new approach for treatment and/or prevention of viral infection.
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MIMETIC is a novel computer program for designing complementary peptides that may interact with a target amino acid sequence of a protein [1]. Complementary peptides targeted to regions regarded to be essential for function of HIV-1 reverse transcriptase (RT) inhibited reverse transcription in vitro [1]. Three complementary peptides (TLMA2993, PSTW1594, and ESLA2340) out of 10 peptides synthesized inhibited RT function in a cell free system. TLMA2993 (N′-TLMALELKGKLLLAGLA PSAFLPLSFPEGL-C′; the name of peptide consists of the one letter code for the first four amino terminal amino acids and its molecular weight) was the strongest of the peptides tested, and 32 μM TLMA2993 could inhibit reverse transcription [1]. TLMA2993 targets the connection domain of RT and we assumed that it could restrict RT function in cells if we transfected cells with the corresponding cDNA. In this work, we generated an artificial cDNA coding for TLMA2993 and demonstrated that transfection of the cDNA induced resistance to HIV-1 infection.

Materials and methods

Preparation and phosphorylation of oligonucleotides. The following oligonucleotides were synthesized: 5′-AATTCCCCACCATGACTTT AATGGCTCTCGAGCTCAA-3′ (TLMA-(i)); 5′-AGGTAAGCTTT

☆ *Abbreviations:* AG promoter, modified chicken β-actin promoter; Amp^r, ampicillin resistance; bp, base pair; CMV-IE, cytomegalovirus immediate early; DNA, deoxyribonucleic acid; EDT, 1,2-ethanedithiol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate isomer; HIV, human immunodeficiency virus; KLH, Hemocyanin, Keyhole Limpet; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; Neo^r, neomycin resistance; NP-40, nonidet P-40; ori, origin of DNA replication; PE, phycoerythrin; PBS, phosphate-buffered saline; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; SV40, simian virus 40; TE, Tris–HCl/EDTA buffer; TFA, trifluoroacetic acid; RT, reverse transcriptase.

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TATTAGCTGGGCTAGCGCCTAGCG-3′ (TLMA-(iii)); 5′-CTT TCTTACCCTTAAGTTTTCCGGAAGGACTTTAGG-3′ (TLMA-(v)); 5′-TTACCTTTGAGCTCGAGAGCCATTAAAGTCATGG TG GGG-3′ (TLMA-(ii)); 5′-AGAAAGCGCTAGGCGCTAGCCC AGC TAATAAAAGC-3′ (TLMA-(iv)); and 5′-AATTCCTAAAGTCCT TCCG GAAAACTTAAGGGTA-3′ (TLMA-(vi)). For gel purification of oligonucleotides, 20 μl of MG dye (80% formamide solution containing 1% xylene cyanol and bromophenol blue) 10 mM NaOH, and 1 mM EDTA were added to the oligonucleotide pellets (approximately 100 μg). After polyacrylamide gel electrophoresis on a sequencing-type gel [14% polyacrylamide (acrylamide:bis-acrylamide = 19:1), 8 M urea, 2 mm thickness], the oligonucleotides, which were detected by ethidium bromide staining, were cut out from the gel and eluted in 1 ml G buffer (0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$, 1 mM EDTA, and 0.1% SDS) at 37 °C for overnight. The eluted oligonucleotides were purified by the column that was made of Whatmann DE-52 resin (diethylaminoethyl cellulose resin, Whatmann BioSystem, Kent, UK) and recovered by ethanol precipitation, and then resuspended in 25 μl water. Oligonucleotides were phosphorylated at a concentration of 100 pmol in a final volume of 20 μl containing polynucleotide kinase buffer (50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, and 5 mM DTT), 10 mM γ-ATP, and 20 U T4 polynucleotide kinase (TaKaRa Biomedicals, Shiga, Japan), incubated at 37 °C for 1 h. The kinase was subsequently inactivated by incubation at 70 °C for 10 min.

Shot-gun ligation method. The pCR2.1 vector (Invitrogen, Carlsbad, California, USA) was cut with *Eco*RI and was dephosphorylated with alkaline phosphatase (TaKaRa Biomedicals). Then shot-gun ligation was performed as described previously (Fig. 1) [2,3]. For shot-gun ligation, 0.5 pmol of the six phosphorylated oligonucleotides was mixed in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 100 mM NaCl, and hybridized for 1 h at 37 °C. One microliter of hybridized DNA and 50 ng of vector fragment were ligated using DNA Ligation Kit Ver. 1 (TaKaRa Biomedicals). The ligation reaction was performed overnight at 16 °C. The nucleotide sequence was determined by the chain termination method using an ABI PRISM 310 genetic analyzer (PE Biosystems, Tokyo, Japan) with M13 forward or reverse primers. The appropriate sequence was excised from TLMA2993/pCR2.1 vector using *Eco*RI, and ligated into pCXN2 vector which was a pCAGGS derivative [4], was cut with *Eco*RI, and dephosphorylated. For linearization, this vector was cut with *Pvu*I in the ampicillin resistant gene.

Cell cultures. U937 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 nM of 2-mercaptoethanol. U937 cells transfected with the TLMA2993/pCXN2/*Pvu*I vector were maintained in the presence of 400 μg/ml G418 (Gibco Life Technologies, Rockville, Maryland, USA). Cultures were kept humid field at 37 °C in 5% CO_2 and passaged every 3–4 days. U937 cells chronically infected with the HIV-1 IIIB strain were maintained under the same conditions.

Stable expression of TLMA2993 peptide. TLMA2993/pCXN2 was transfected into U937 cells by electroporation using a Gene Pulser (Bio-Rad, Hemel Hempstead, UK). Twenty micrograms of plasmid DNA (TLMA2993/pCXN2/*Pvu*I digested) in TE was added to 5×10^6 cells in 400 μl RPMI 1640 medium (without antibiotics) in a 0.4 cm cuvette (Bio-Rad), and given a single pulse at 960 μF, 250 V [5]. The cells were added to 25 ml medium, left to recover overnight in normal medium, and then grown in selective medium (400 μg/ml G418). The cells were dispensed into a 24-well plate. Individual clones from a mixed population of stably transfected cells were isolated by using micropipette.

Screening by genomic PCR and RT-PCR. The oligonucleotides synthesized were as follows: 5′-TCCTACAGCTCCTGGGCAAC-3′ (sense strand), 5′-GAGCCAGGGCATTGGCCACA-3′ (antisense strand). These oligonucleotides were able to amplify the region that

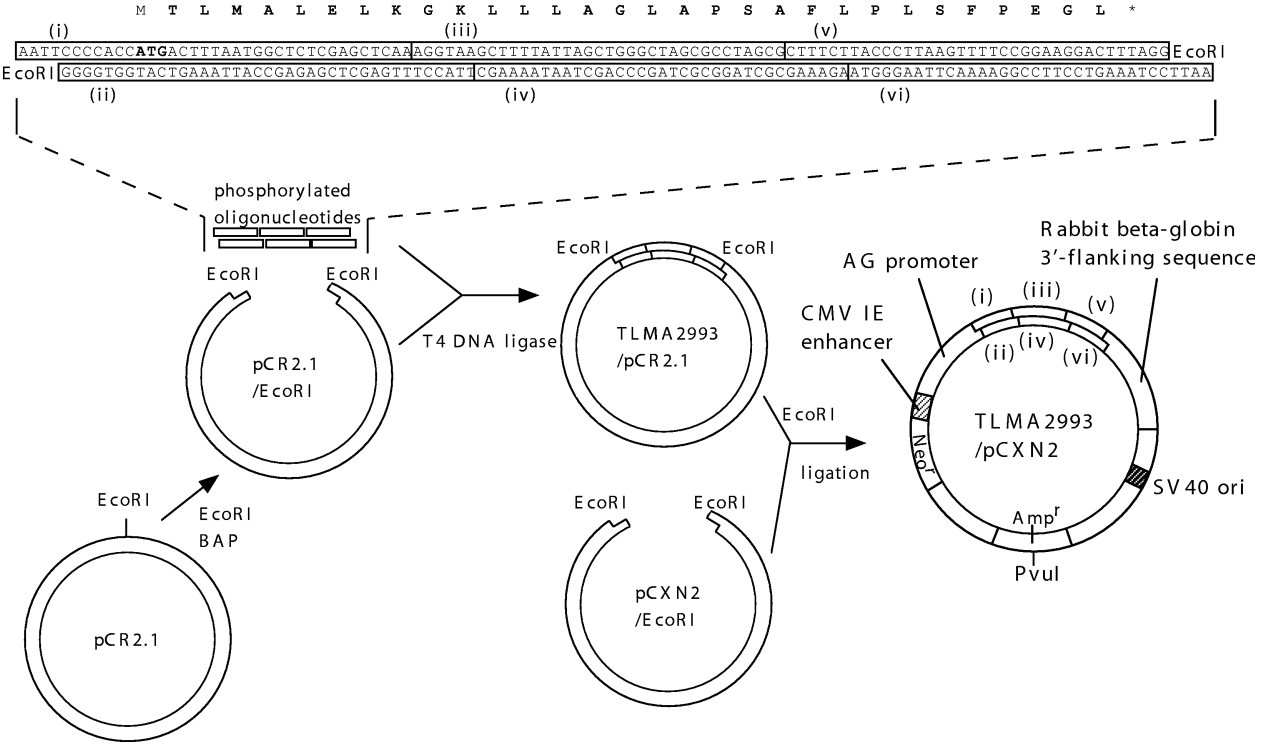

Fig. 1. Outline of the shot-gun ligation method. The vectors were constructed as described in Materials and methods. pCR2.1/*Eco*RI was employed for cloning of six overlapping synthetic oligonucleotides ((i)–(vi), in the upper part of the figure) that constructed the TLMA2993 peptide to which was added an additional N-terminal methionine.

was inserted into the *Eco*RI site of the pCXN2 vector. On genomic DNA isolated using standard methods, PCR amplification was performed at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and ending with 72 °C for 7 min using a Gene Amp PCR System 9700 (PE Biosystems).

To detect TLMA2993 mRNA, RT-PCR was performed on total RNA from TLMA2993 transfected cells. Following reverse transcription, PCR amplification was performed at 94 °C for 1 min, followed by 30 cycles of 94 °C for 5 s, 50 °C for 15 s, and 72 °C for 1 min, and ending with 72 °C for 2 min TLMA-(i) and TLMA-(vi) oligonucleotides were used in this RT-PCR.

Flow cytometric analysis of surface protein. Cells were harvested and washed in PBS, and resuspended at 2×10^6 cells/ml. Aliquots (100 μl) were plated in a V-bottomed 96-well plate (2×10^5 cells/well). Plate was centrifuged at 1000 rpm at 4 °C for 5 min, supernatant was removed, and the cells were gently resuspended in 50 μl of anti-CD4-PE, anti-CXCR4-PE (Pharmingen, San Diego, California, USA), or anti-CCR5-FITC (R&D Systems, Minneapolis, Minnesota, USA), and the plates were placed on ice for 30 min. The cells were washed twice in PBS, resuspended in FACSFlow Sheath Fluid (Becton–Dickinson, San Jose, California, USA), and then analyzed by FACS Calibur (Becton–Dickinson) [6].

Co-cultivation experiment. 2×10^4 U937/TLMA-15, U937/TLMA-18 or U937/N2 cells were mixed with 1×10^2 (200:1) or 40 (500:1) HIV-1 infected U937 cells (U937/IIIB), and the mixtures were cultured in 1.0 ml RPMI1640 containing 10% FCS in a 24-well plate, as described previously [7,8]. Every fourth day, 0.5 ml of the cultures was collected and the percentage of infected cells was determined using a Coulter Colon KC-57-FITC anti-p24 monoclonal antibody (Coulter, Hialeah, Florida, USA) following the manufacturer's protocol for staining p24 and the percentage of HIV infected cells was determined using FACS Calibur. Eighty microliters of residual cell was transferred to 920 μl of culture medium [7,8].

Peptide synthesis. Peptides were synthesized by the solid phase method with F-moc chemistry using an AMS peptide synthesizer (ABIMED, Langenfeld, Germany). These were then cleaved from the resin, with the concomitant removal of side-chain protecting groups by treatment with trifluoroacetic acid (TFA), 80%; thioanisol; 12%; 1,2-ethanedithiol (EDT), 6%; and *m*-cresol, 2%. Peptides were then purified by high performance liquid chromatography on a reversed C18 column with 0.1% TFA/water–acetonitrile. All peptides were confirmed using time of flight mass spectrometry on a KOMPACT MALDI II (Kratos-Shimadzu, Kanagawa, Japan) [9].

Production of antiserum against TLMA2993. The carrier protein, Hemocyanin, Keyhole Limpet (KLH; Calbiochem, San Diego, California, USA) was linked to *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Pirece, Rockford, Illinois, USA) forming an MB/KLH conjugate using standard methods. After column purification, the MB/KLH conjugate was cross-linked to the cysteine-containing synthetic peptide corresponding to the N-terminal 10 amino acids of TLMA2993 peptide (N′-TLMALELKGKC-C′, termed TLMA(Nt) peptide), suspended in approximately 80 μg of peptide/carrier conjugate in PBS, and mixed with complete Freund's adjuvant. Three rabbits were immunized subcutaneously with this mixture and 2 weeks later, each rabbit was boosted with 50 μg of the conjugate in PBS in incomplete Freund's adjuvant. Additional boosts were administered at the 5th and 21st weeks. Rabbits were bled 1 week after the last immunization to obtain antipeptide serum.

Competitive ELISA. Fifty microliters of TLMA(Nt) peptide (1 μg/ml), corresponding to the N-terminal of TLMA2993 peptide, in PBS was added to a Falcon 3911 96-well U-bottomed plate (Becton–Dickinson Labware, Bedford, Massachusetts, USA) and incubated at 4 °C overnight. After washing with 0.05% Tween 20 in PBS (PBST) five times, wells were blocked using 200 μl PBS containing 2% BSA and incubated at room temperature for 2 h, followed by washing with PBST five times. To generate a standard curve, U937/N2 cells (electroporation with empty vector) lysed by TNE buffer (5×10^6 cells/1 ml TNE buffer (10 mM Tris–HCl, pH 7.9, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, and 10 μg/ml aprotinin)) were mixed with serial dilutions of TLMA2993 peptide (final concentrations were 32, 12, 6.0, 3.0, 1.5, 0.75, and 0.38 μM). Rabbit serum (final concentration 1:1000) was mixed with U937/N2 cell lysate (2.5×10^5 cells/well). A standard inhibition curve was generated for dose dependent inhibition of the ELISA reaction by the peptide mixed with the control cell lysate. U937/TLMA-15 and 18 cells were lysed in the same manner and mixed with rabbit serum at the same ratio of U937/N2 lysate. Sixty microliters of each mixture was added to the micro plate, incubated at room temperature for 2 h, and then washed with PBST five times. Peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, Alabama, USA) was then added to the plate and incubated at room temperature for 1 h. After washing, peroxidase enzyme activity was detected by addition of a solution containing 0.015% hydrogen peroxide and 0.04% *O*-phenylenediamine followed by incubation for 5–10 min. Finally, 2 N H_2SO_4 was added to stop the reaction and the absorbance at 492 nm was determined. The immunoreactive peptide in the samples was determined using a standard curve.

Detection of HIV-1 DNA using PCR. TLMA2993 transfected cells (2×10^5 cells/0.5 ml) were mixed with 0.5 ml of the culture supernatant of HIV-1 IIIB chronically infected U937 cells ($10^{3.2}$ $TCID_{50}$/100 μl) and incubated for 1 h at 37 °C with shaking. After shaking, cells were incubated in a CO_2 incubator and collected at 24 and 48 h. The amount of HIV-1 DNA was detected by PCR at 24 and 48 h after HIV-1 infection. After fixation of HIV-1 infected cells with 1% paraformaldehyde for 1 h, genomic DNAs were prepared using standard methods. The following oligonucleotides were synthesized for PCR amplification of 180 bp of the HIV-1 LTR region [10]: 5′-GGTCTCTCTGGTTAGACCAGAT-3′ (RU5-5′ primer), 5′-CTGCTAGAGATTTTCCACACTG-3′ (RU5-3′ primer). PCR amplification was performed using 50 ng DNA template from cells to be tested, at 94 °C for 1 min, followed by 30 cycles of 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s, and ending with 72 °C for 5 min. PCR products were analyzed on a 4% polyacrylamide gel. Parallel reactions for quality control of the DNA were shown by amplification of the β-actin gene. The annealing temperature was changed to 65 °C. Synthesized oligonucleotides for amplification of the β-actin gene were 5′-GAAATCGTGCGTGACATTAAG-3′ (β-actin 5′ primer) and 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3′ (β-actin 3′ primer) [11].

Results

Strategy of shot-gun ligation

TLMA2993 is an artificially generated peptide and produced the gene which coding TLMA2993 artificially. Then shot-gun ligation method was performed by using synthetic oligonucleotides that overlap complementarily. Synthetic DNA molecules that contained both the appropriate 5′ and 3′ sticky ends would allow circularization of the vector DNA during the ligation reaction and created transformants in *Escherichia coli*. The strategy used is shown in Fig. 1 and Materials and methods. The pCR2.1 vector was cut with *Eco*RI, dephosphorylated, and precipitated with ethanol. Sets of six overlapping oligonucleotides corresponding to TLMA2993 peptide that was added to additional N-terminal methionine and generating *Eco*RI sticky ends were synthesized. The oligonucleotides were phosphorylated by T4 polynucleotide kinase, hybridized, ligated with the

vector DNA, and used to transform *E. coli*. Progeny colonies from shot-gun ligation method were screened by nucleotide sequencing, which was determined by the chain termination method using ABI PRISM 310 genetic analyzer with M13 universal or reverse primers. The clones showed apparently correct sequences. Then appropriate sequence was cut out using *Eco*RI and ligated into pCXN2/*Eco*RI vector. The suitable direction was checked by nucleotide sequencing. To produce stable transfectant, it was necessary to linearize the vector. TLMA2993/pCXN2 vector was cut with *Pvu*I on the ampicillin resistant gene before performing the electroporation.

Characteristic of transfectants

Stable clones of U937 cells expressing TLMA2993 peptide were generated by electroporation of the linearized TLMA2993/pCXN2. Four clones (U937/TLMA-8, 15, 18, and 20) out of 20 transfectants were selected and cDNA expression was confirmed by genomic PCR and RT-PCR. For a control, U937/N2, which was transfected with empty vector, was established under the same conditions.

Since infectivity of HIV-1 is influenced by the amount of CD4 and chemokine receptors such as CXCR4 and CCR5 which function as HIV-1 receptor and co-receptors on cell surfaces, we determined the level of these molecules on transfected cells. On cytometric analysis, cell surface expression of CCR5 was significantly lower in U937/TLMA-8 cell than in others, and CXCR4 expression was a little higher in U937/TLMA-8 and U937/TLMA-20 cells, although CD4 expression was almost the same in all clones (data not shown). The expression patterns of CCR5 and CXCR4 on U937/TLMA-15, U937/TLMA-18, and U937/N2 were close to the same level. Therefore, U937/TLMA-15, U937/TLMA-18, and U937/N2 were chosen and their resistance to HIV-1 infection was evaluated. The concentrations of TLMA2993 peptide in the U937/TLMA-15 and U937/TLMA-18 determined by the competitive ELISA were 1.8 and 1.3 μM, respectively.

Anti-HIV infectivity assay

To evaluate the ability of TLMA2993 peptide to render cells resistant to HIV-1 infection, 2×10^4 U937/TLMA-15, U937/TLMA-18 or U937/N2 cells were mixed with 1×10^2 (200:1, Fig. 2A) or 40 (500:1, Fig. 2B) HIV-1 IIIB chronically infected U937 cells. Following co-cultivation, we assessed the percentage of HIV-1 infected cells with a Coulter Colon KC-57-FITC anti-

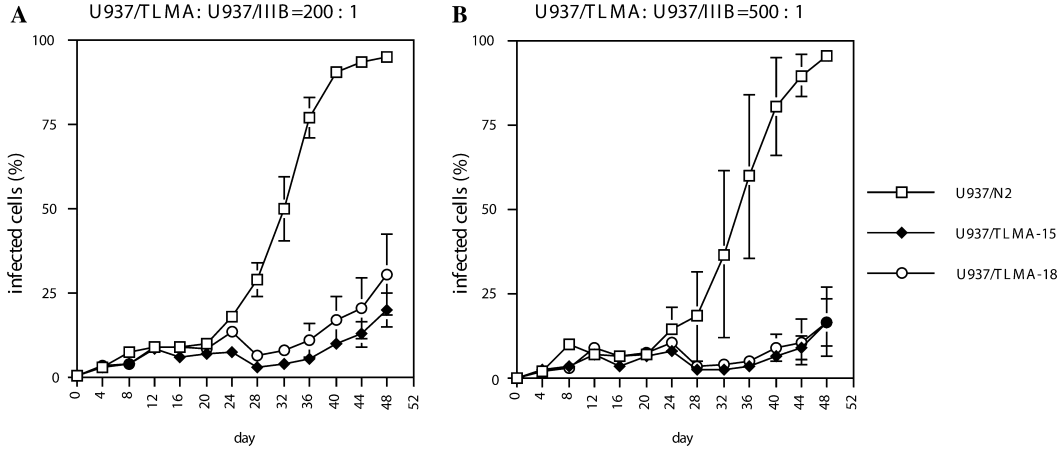

Fig. 2. Inhibition of HIV-1 spread in a mixed culture. TLMA2993 transfected cells (2×10^4) were mixed with 1×10^2 (A) or 40 (B) HIV-1 IIIB-infected U937 cells in 24-well plates with 1 ml medium. U937/TLMA-15 (♦), U937/TLMA-18 (○), and U937/N2 (□) are shown. The number of HIV-infected cells was assessed as described in Materials and methods. The values shown represent means of assays performed in triplicate (SD shown).

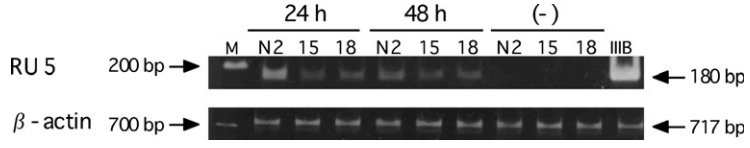

Fig. 3. PCR amplification of HIV-1 DNA in the LTR region. TLMA2993 transfected cells (15 and 18) and control cells (N2) were infected with HIV-IIIB. After cultivation for 24 and 48 h, cells were subjected to PCR amplification of 180 bp of the HIV-1 LTR region. Parallel reactions for quality control of the DNA are shown by amplification of the β-actin gene. Size markers are in the left lane. The right lane is DNA extracted from HIV-IIIB clonically infected U937 cells.

p24 mAb according to the manufacturer's protocol. The percentage of infected cells on day 40 was 90.6% in U937/N2 cells, whereas in U937/TLMA-15 and U937/TLMA-18 cells, these values remained at only 10.2% and 17.0%, respectively (Fig. 2A). By day 48, the percentage of infected U937/N2 cells was 95.4%, whereas U937/TLMA-15 and U937/TLMA-18 cells remained at 16.4% and 16.6%, respectively (Fig. 2B).

Detection of HIV-1 DNA using PCR

TLMA2993 transfected cells were mixed with 0.5 ml of the culture supernatant of HIV-1 IIIB infected U937 cells and incubated for 1 h at 37 °C in a CO_2 incubator with shaking. After incubation, cells were collected at 24 and 48 h. The amount of HIV-1 DNA detected by PCR at 24 and 48 h after HIV-1 infection was significantly suppressed in U937/TLMA-15 and U937/TLMA-18 while that of U937/N2 was at an appreciable level (Fig. 3).

Discussion

Since TLMA2993 significantly inhibited reverse transcription in a cell free system [1], we designed, and synthesized an artificial cDNA to generate TLMA2993 in the transfectants. As expected, the transfectants became resistant to HIV-1 infection. The amount of TLMA2993 peptide detected by competitive ELISA was 1.8 μM for U937/TLMA-15 and 1.3 μM for U937/TLMA-18, and U937/TLMA-15 showed a higher resistance than U937/TLMA-18 (Figs. 2 and 3). It is clear that the data shown in Figs. 2 and 3 reflected the results of competitive ELISA. Since the levels of expression of CD4, CCR5, and CXCR4 were essentially the same among the cells, resistance could be correlated with the amount of TLMA2993 expressed and was dose dependent.

Inhibition of HIV-1 infection in the transfectants was actually due to suppression of RT function, since generation of HIV-1 DNA at an early stage of infection was suppressed (Fig. 3). It is likely that the current dose of nucleoside analogues or non-nucleoside drugs could be lowered by combination with TLMA2993 peptide or other complementary peptides of RT. A stronger effect on HIV-1 infection would be expected if the three kinds of complementary peptides were combined. It will be necessary to also test peptides similar to TLMA2993 such as PSTW1954 and ESLA2340 that are other RT inhibitors [1], and to confirm their effects in the cell. Complementary peptides of RT will have a potential to cure HIV-1 infected patients and this complementary peptide anti-viral therapy provides a novel approach.

The method described here may be applicable to the regulation of any intracellular functional protein. Complementary peptides such as TLMA2993 can be designed using a program such as MIMETIC, and these may be expressed using an artificial cDNA as a means of regulating target molecules in cells.

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