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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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Keywords: Complementary peptide; Transfection; HIV-1; Reverse transcriptase; Connection domain; Shot-gun ligation method

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

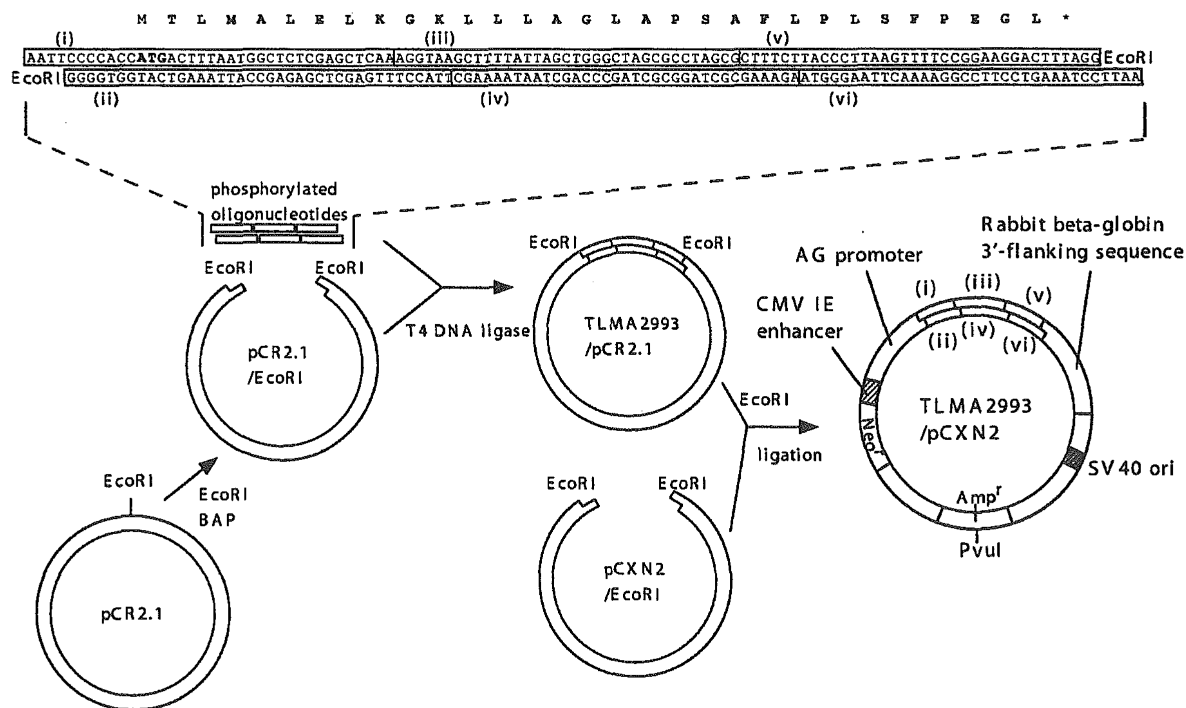

Fig. 1. Outline of the shot-gun ligation method. The vectors were constructed as described in Materials and methods. pCR2.1/*Eco*RI was employed for cloning of six overlapping synthetic oligonucleotides ((i)–(vi), in the upper part of the figure) that constructed the TLMA2993 peptide to which was added 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′-GGTCTCTCTGGTTA GACCAGAT-3′ (RU5-5′ primer), 5′-CTGCTAGAGATTTTCCAC ACTG-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′-GAAATCGTGCGTGA CATTAAG-3′ (β-actin 5′ primer) and 5′-CTAGAAGCATTTGC GGTGGACGATGGAGGGGCC-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-

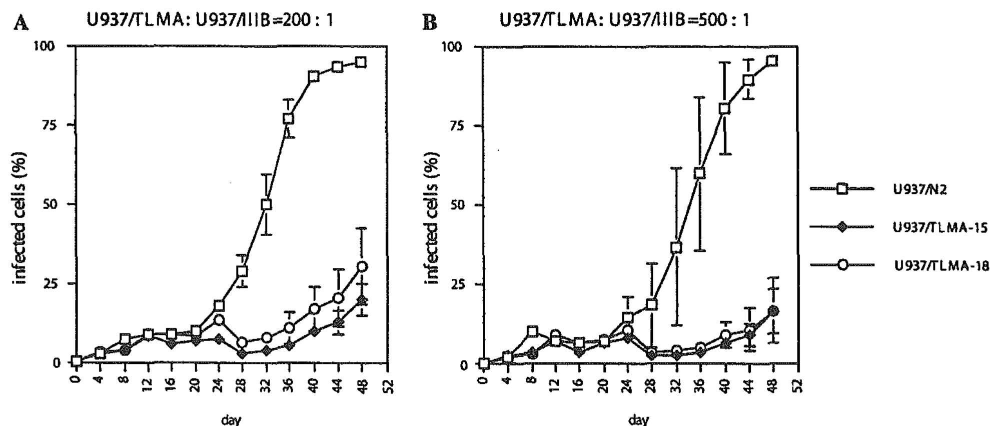

Fig. 2. Inhibition of HIV-1 spread in a mixed culture. TLMA2993 transfected cells (2×10^4) were mixed with 1×10^2 (A) or 40 (B) HIV-1 IIIB-infected U937 cells in 24-well plates with 1 ml medium. U937/TLMA-15 (◆), U937/TLMA-18 (O), and U937/N2 (□) are shown. The number of HIV-infected cells was assessed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

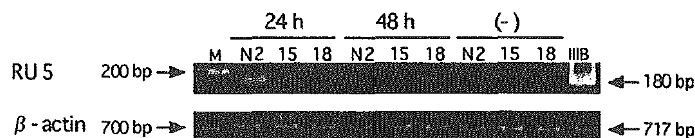

Fig. 3. PCR amplification of HIV-1 DNA in the LTR region. TLMA2993 transfected cells (15 and 18) and control cells (N2) were infected with HIV-IIIB. After cultivation for 24 and 48 h, cells were subjected to PCR amplification of 180 bp of the HIV-1 LTR region. Parallel reactions for quality control of 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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Delayed HIV-1 Infection of CD4+ T Lymphocytes from Therapy-Naïve Patients Demonstrated by Quantification of HIV-1 DNA Copy Numbers

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***Abstract*: Measuring the amount of HIV-1 DNA in infected cells is important to estimate the size of the viral reservoir in patients. However, the clinical impact of the intracellular viral DNA level remains unclear. The present study examines the clinical significance of the HIV-1 DNA level in peripheral $CD4^+$ T lymphocytes from 21 therapy-naïve patients. HIV-1 DNA levels in purified peripheral $CD4^+$ T lymphocytes were measured by the real-time PCR method using the Roche LightCycler system that can detect 200 copies/10^6 cells. We detected intracellular HIV-1 DNA in 15 (71.4%) of 21 patients at levels ranging from 270 to 98,120 copies/10^6 $CD4^+$ cells, with a median of 2,220 copies/10^6 cells. We also found HIV-1 DNA that was below the detection limit in the remaining 6 patients, although 8,800–150,000 copies/ml of HIV-1 RNA were detected in plasma. Circular HIV-1 DNA was not detected in 5 of 6 cases, suggesting that reverse transcription in $CD4^+$ T lymphocytes of these cases was not active. Thus, delayed HIV-1 infection of $CD4^+$ T lymphocytes was demonstrated in these patients. The level of HIV-1 DNA in peripheral $CD4^+$ T lymphocytes indicates the clinical status of therapy-naïve patients.**

Key words: **Delayed HIV-1 infection, Therapy-naïve, Real-time PCR, HIV-1 DNA**

Human immunodeficiency virus type 1 (HIV-1) efficiently and continuously replicates itself after inserting its genome into the DNA of host cells. Such active viral replication correlates directly with disease progression and patient survival (11). Therefore, the HIV-1 RNA level in plasma directly reflects viral replication and has become a powerful prognostic tool (14).

Highly active antiretroviral therapy (HAART) that basically includes combinations of nucleoside or non-nucleoside inhibitors of reverse transcriptase (RT) and a protease inhibitor(s) can significantly reduce plasma HIV-1 RNA to below detectable levels. However, even after years of HAART treatment, cells harboring replication-competent HIV-1 can still persist in the blood and lymphoid tissues (3–5, 8, 9, 18, 19). This persistent reservoir of infected cells is the major impediment to HIV-1 eradication. Therefore, it is important to estimate the viral reservoir and to study its dynamics by measuring intracellular HIV-1 DNA levels. However, the clinical significance of intracellular HIV-1 DNA levels remains unclear.

Real-time PCR can treat many samples in a short period, making it useful for studying intracellular HIV-1 persistence (6, 7). In this report, we first validated the real-time PCR method and then successively measured intracellular HIV-1 DNA levels in 21 therapy-naïve patients. We specifically aimed to determine the status of HIV-1 infection in patients carrying a detectable plasma viral load, but whose $CD4^+$ T lymphocytes were minimally infected.

Materials and Methods

Patients. Twenty-one therapy-naïve HIV-1-infected patients who underwent initial consultation at Nagoya Medical Center, Japan, were enrolled in this study. The

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Abbreviations: β2M, β_2-microglobulin; HAART, highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase.

quantification of HIV-1 was performed after informed consent was obtained.

Measurements of plasma viral load and CD4 cell counts. Viral load was measured using an Amplicor HIV-1 monitor v1.5 system (Roche Diagnostics, Tokyo). CD4 cell counts were performed by flow cytometry with FACSCalibur (Becton Dickinson, Tokyo) using anti-CD4 antibody (DakoCytomation, Kyoto, Japan).

Purification of CD4-positive lymphocytes and DNA extraction. $CD4^+$ lymphocytes were isolated by StemSep column chromatography (Stem Cell Technologies, Vancouver, BC, Canada). Collected cells were washed and resuspended in phosphate-buffered saline. DNA was extracted using QIAamp DNA Blood Kits (QIAGEN, Tokyo).

Preparation of HIV-1 DNA and β2M DNA assay standards. A standard HIV-1 plasmid (pUC-IIIB) was constructed by cloning one copy of HIV-1 IIIB without LTR into pUC118 (TaKaRa, Shiga, Japan). A human β_2-microglobulin (β2M) standard plasmid (pGEM-β2M) was constructed by cloning one copy of β2M exon 2 to pGEM-T (Invitrogen, Tokyo).

Quantification of HIV-1 DNA by real-time PCR. We designed PCR primers and a *Taq*Man probe for HIV-1 DNA based on the HIV-1 subtype B consensus sequence (database of Los Alamos National Laboratory). The amplification primers were located in the *gag* region: forward primer (Gag 1), 5′-CAAGCAGCCATGCAAATGTT-3′ and reverse primer (Gag 2), 5′-GCATGCACTGGATGCAATCT-3′. The *Taq*Man probe has the sequence 5′-FAM-TCCATTCTGCAGCTTCCTCATTGATG-TAMRA-3′. Copy numbers of the β2M gene were determined using the primers, 5′-CAGCAAGGACTGGTCTTTCTATCTCT-3′ and 5′-ACCCCACTTAACTATCTTGG-3′, with the *Taq*Man probe, 5′-FAM-CACTGAAAAAGATGAGTATGCCTGCCGTGT-TAMRA-3′.

Real-time PCR proceeded using an LC Fast Start DNA master mix hybridization probe kit (Roche Diagnostics). The PCR mixtures contained 60 ng of DNA extracts, 2 μl of DNA master mix, 5 mM $MgCl_2$, primers (500 nM each) and the *Taq*Man probe (400 nM) in a total volume of 20 μl. Cycling parameters consisted of denaturation for 10 min at 95 C followed by 45 cycles of 10 sec at 95 C and 30 sec at 60 C. The automated LightCycler system performed the amplification, as well as data acquisition and analysis.

Determination of HIV-1 subtypes and tropisms. We determined the nucleotide sequence of the *V3* region of the *env* gene to classify the HIV-1 subtype and tropism as described (10, 17).

Sequencing HIV-1 gag *region.* Nucleotides containing the region amplified by real-time PCR were amplified by nested PCR using the external primers gag03 (5′-AAAACATATAGTATGGGCAA-3′) and gag05 (5′-GGGCTATACATTCTTACTAT-3′) and the internal primers gag06 (5′-GATAGAGGTAAAAGACACCAA-3′) and gag04 (5′-TAGGTGGATTGTTTGTCATC-3′).

The DNA in both reactions was denatured for 5 min at 95 C followed by 30 cycles of 30 sec at 95 C, 30 sec at 50 C and 1 min at 72 C and a final extension for 7 min at 72 C. The DNA was amplified in a total volume of 50 μl containing 1× LA *Taq* buffer (TaKaRa), 2.5 mM $MgCl_2$, 0.1 mM each dNTP, 400 nM each primer, and 1 U LA *Taq* DNA polymerase (TaKaRa). Genomic DNA (60 ng) was amplified by the first PCR, and 5 μl of this mixture was applied to the nested reaction.

Both sense and antisense strands of PCR products were sequenced directly using BigDye Terminator Sequencing Kits and an ABI PRISM 310 automatic sequencer (Applied Biosystems, Tokyo).

The sequences of the PCR products were deposited in the DNA Data Bank of Japan (accession numbers AB154280 through AB154297).

Detection of unintegrated circular HIV-1 DNA. Unintegrated circular HIV-1 DNA was amplified by nested PCR using C1R1 (5′-GACCTCAGGTACCTTTAAGA-3′) and C1R2 (5′-GCTTAATACTGACGCTCTCGC-3′) primers in the first reaction, and C3R1 (5′-GGGAGCTTTAGATCTTAGCC-3′) and C3R2 (5′-CCTTCTAGCCTCCGCTAGTC-3′) primers in the nested reaction.

The conditions and PCR mixture components were identical to those used for HIV-1 gag sequencing, except *Taq* DNA polymerase (Roche Diagnostics) was substituted for LA *Taq* polymerase.

Results

Accuracy, Reproducibility and Sensitivity of HIV-1 DNA Quantification Using LightCycler

Table 1 shows the validation data obtained using latently HIV-1-infected ACH2 cells containing one provirus per cell. The interassay CV% was 7.1, 11.7, 50.7 and 71.9 at 10^4, 10^2, 5 and 2 copies, respectively. The accuracy (%) values of the corresponding experiments were 100.4±7.1, 101.2±11.9, 101.2±51.3 and 69.7±50.1, respectively. The intra-assay reproducibility of HIV-1 copy numbers was determined as shown in Table 1. When pUC-IIIB standard plasmids were the HIV-1 DNA source instead of ACH2, the accuracy and reproducibly of HIV-1 DNA measurements were identical (data not shown). These results show that quantification of HIV-1 DNA with LightCycler can be performed with high sensitivity and reproducibility. Data were normalized as copies/10^6 cells by measuring copy

Table 1. Accuracy and reproducibility of real-time PCR assay using LightCycler system

HIV-1 DNA	Intra-assay (n=5)			Interassay (n=15)		
(copies/10^4 cells)	Mean±SD	CV%	Accuracy (%)	Mean±SD	CV%	Accuracy (%)
10,000	9,548±321	5.2	95.5±3.2	10,048±712	7.1	100.4±7.1
1,000	944±48	5.1	94.4±4.8	930±67	7.2	93.0±6.7
100	94±5	4.7	94.0±4.5	101±12	11.7	101.2±11.9
50	49±7	13.0	98.4±4.5	53±7	13.1	106.9±14.0
10	11±1.2	14.0	106.9±12.0	12±3.1	25.9	118.7±30.8
5	4.6±1.8	38.3	91.8±35.2	5.1±2.6	50.7	101.2±51.3
2	1.2±0.5	45.3	58.6±26.6	1.4±1.0	71.9	69.7±50.1

Table 2. HIV-1 DNA level of 21 therapy-naïve HIV-1-infected patients

Patient No.	CD4 cell count (cells/μl)	Plasma HIV-1 RNA (copies/ml)	HIV-1 DNA[a] (copies/10^6 cells)	Detection of circular HIV-1 DNA	Subtype
1	602	1,200	1,070	−	B
2	298	1,600	270	+[c]	B
3	388	8,800	<DL[b]	−	B
4	350	13,000	<DL[b]	−	C
5	292	18,000	<DL[b]	−	B
6	295	20,000	2,440	+[d]	E
7	542	33,000	3,600	−	B
8	329	35,000	<DL[b]	−	B
9	715	44,000	450	+[d]	B
10	281	50,000	10,800	+[d]	B
11	441	54,000	1,440	−	B
12	283	70,000	2,220	+[d]	B
13	330	92,000	300	−	B
14	619	93,000	<DL[b]	−	B
15	116	110,000	15,290	+[d]	B
16	98	120,000	98,120	+[c]	B
17	229	150,000	<DL[b]	+[c]	B
18	527	210,000	1,930	+[d]	B
19	49	210,000	2,870	+[d]	B
20	307	250,000	1,620	+[d]	B
21	21	430,000	33,240	+[d]	B
Median (range)	307 (21–715)	54,000 (1,200–430,000)	2,220 (<DL–98,120)		

[a] Average values of HIV-1 DNA assayed in duplicant.
[b] Below limits of detection.
[c] 1 and 2LTR circular DNA.
[d] 1LTR circular DNA.

numbers of the β2M gene since two β2M copy numbers correspond to one cell. Since 10^4 cells were usually used in one assay, we defined the lower limit of detection as 200 copies/10^6 cells.

Total HIV-1 DNA Copy Numbers in CD4+ T Lymphocytes from Therapy-Naïve Patients

We determined the intracellular HIV-1 DNA in 21 therapy-naïve HIV-1-infected patients. Fifteen (71.4%) of the 21 patients had HIV-1 DNA levels ranging from 270 to 98,120 copies/10^6 $CD4^+$ cells, with a median of 2,220 copies/10^6 cells (Table 2). Amounts of HIV-1 DNA were below the limits of detection in the remaining 6 patients (patients 3, 4, 5, 8, 14 and 17).

The HIV-1 subtypes were B (n=19), C (n=1, patient 4) and E (n=1, patient 6).

Matching of Primers and Taq*Man Probe*

To eliminate the possibility that the low HIV-1 copy number was underestimated because of mismatching, we analyzed the nucleotide sequences of HIV-1 DNA from 6 patients containing the same regions as the primers and the *Taq*Man probe region (Fig. 1). The Gag 1 region corresponding to the forward primer did not contain any mutations in patients 3, 5 and 8 with undetectable levels of HIV-1 DNA. However, we iden-

tified one mutation (C→T) in the center of this region in patients 4, 14 and 17. In the Gag 2 region, all patients had some mutations, however, none of them were located at the 3′-OH end. In the *Taq*Man probe region, we identified no mutations in 4 of 6 patients (4, 8, 14 and 17), and one in the remaining 2 patients (3 and 5). We estimated that these mutations were not critical to the real-time PCR reaction because of their positions. Sequencing the HIV-1 DNA from patients with high levels revealed the same or similar nucleotide mutations. We concluded that the low levels of HIV-1 DNA found in the 6 patients were not false-negative values.

Relationship between Intracellular HIV-1 DNA Levels and CD4⁺ Cell Count or Plasma Viral Load

A negative relationship was observed between intracellular HIV-1 DNA levels and CD4⁺ cell count (R=0.483). The tendency was the same (P=0.033, Kruskal-Wallis test) when the 21 patients were classified into 3 groups (L, <200; IM, 200–350 and H, >350 cells/μl) (Table 3). On the other hand, a positive relationship between intracellular HIV-1 DNA levels and plasma viral load was observed although the association was quite weak (R=0.287). The Kruskal-Wallis test showed no correlation between them (P=0.125).

Detection of Unintegrated Circular HIV-1 DNA

We further investigated whether T lymphocytes from 6 patients were minimally infected with HIV-1 by detecting circular HIV-1 DNAs that are sensitive markers of early HIV-1 infection (2, 12, 13). Figure 2 shows the results of agarose gel electrophoresis. We detected

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A) HIV-1 DNA level <DL
                  Gag 1                             TaqMan Probe                    Gag 2
B consensus  CAAGCAGCCATGCAAATGTT AAAAGAGAC CATCAATGAGGAAGCTGCAGAATGGGA T AGATTGCATCCAGTGCAGGC
patient 3    .................... ......A.. A.......................... . ........C...........
patient 4*   ........T........... ......... ........................... . .............C......
patient 5    .................... ......C.. ............G.............. C ...G.A...........T..
patient 8    .................... ......... ........................... . .....A........A..T..
patient 14   ........T........... ......A.. ........................... . .............C......
patient 17   ........T........... ...G..A.. ........................... . .....A...........T..

B) HIV-1 DNA level >DL
                  Gag 1                             TaqMan Probe                    Gag 2
B consensus  CAAGCAGCCATGCAAATGTT AAAAGAGAC CATCAATGAGGAAGCTGCAGAATGGGA T AGATTGCATCCAGTGCAGGC
patient 1    .................... ......... ........................... . ...G.............T..
patient 2    .................... ...G..... ........................... . ...A.............T..
patient 6**  ........T........... ......... ........................... . ...G.............T..
patient 9    .................... ......... ........................... . ........C........T..
patient 10   .................... ......A.. A.......................... . ........C...........
patient 11   .................... ......A.. T.......................... . ....................
patient 12   .................... ......... ............G.............. . .................T..
patient 13   .................... ......A.. T.......................... . ........C........T..
patient 15   .................... ......... ........................... C .................T..
patient 18   .................... ......... ........................... . ...G.............T..
patient 20   .................... ......... ........................... . ...G.............T..
patient 21   .................... ......... ........................... . .................T..
```

Fig. 1. Nucleotide sequence of amplified region by real-time PCR. Sequences of forward primer (Gag 1), *Taq*Man probe, and reverse primer (Gag 2) used for HIV-1 DNA quantification are boxed. Mutations in these areas are shown by capital letters corresponding to those of mother sequences. *: subtype C, **: subtype E.

Table 3. Levels of HIV-1 DNA according to CD4⁺ cell count or plasma VL category

Categories	No. of patients tested	HIV-1 DNA (copies/10⁶ CD4⁺ cells)
CD4⁺ cell count (cells/μl)		
<200	4	24,270 (2,870–98,120)*
200–350	10	1,920 (<DL–10,800)*
>350	7	1,440 (<DL–3,600)*
Plasma HIV-1 RNA (copies/ml)		
<50,000	9	1,070 (<DL–3,600)**
50,000–100,000	5	1,830 (<DL–10,800)**
>100,000	7	9,080 (<DL–98,120)**

*: P=0.033, **: P=0.125 (Kruskal-Wallis test).

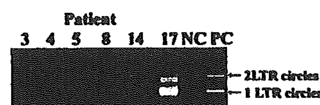
one LTR-circular HIV-1 DNA with or without two LTR-circular types in 11 of 15 patients in whom real-time PCR showed that intracellular HIV-1 DNA levels were detectable. However, no circular HIV-1 DNA was detected in the remaining 4 patients who were judged positive by real-time PCR (Table 2). In contrast, as predicted, no circular form HIV-1 DNA was detected among the patients who were judged negative by real-time PCR except patient 17. Circular HIV-1 DNA was detected at a rate of 100% in the group with the highest plasma viral load (>10^5 copies/ml), with the lowest CD4$^+$ cell count (<200 cells/µl), and with the highest intracellular HIV-1 DNA levels (>10^4 copies/10^6 cells) (Table 4).

Discussion

We measured HIV-1 DNA copy numbers in CD4$^+$ T lymphocytes from 21 therapy-naïve patients using real-time PCR with the Roche LightCycler system. The lower limit of detection was 200 copies/10^6 cells. The intracellular HIV-1 DNA copy numbers ranged from below detectable levels to 98,120 copies/10^6 cells. This distribution was similar to those reported by Désiré et al. (7), Andreoni et al. (1) and Riva et al. (15). Although the number of patients studied might be insufficient to statistically determine an association between plasma HIV-1 viral load and levels of HIV-1 DNA, we identified a weak positive relationship. The key point was the demonstration of the presence of CD4$^+$ T lymphocytes containing a minimal level of HIV-1 DNA in 6 of 21 therapy-naïve patients despite high levels of viral load ranging from 8,800 to150,000 copies/ml. Thereupon, we evaluated the tropism of plasma HIV-1 RNA of these viruses by sequencing the *env V3* region. In all cases, the determined tropism was M-tropic suggesting that viral replication is actively ongoing in target cells of M-tropic HIV-1. In contrast, HIV-1 infection in CD4$^+$ T lymphocytes was not widely established. Current belief is that M-tropic HIV-1 predominantly replicates in patients at the first stage of infection, followed by an increase in T-tropic HIV-1 variants as reported by Schuitemarker et al. (16). According to this model, the very low level of HIV-1 DNA in the CD4$^+$ T lymphocytes found in this study might reflect the infection stage where almost all CD4$^+$ T lymphocytes remain free from HIV-1. Circular HIV-1 DNA was undetectable in most such patients, supporting this notion because this molecular species of HIV-1 DNA reflects active reverse transcription and replication.

From this viewpoint, the HIV-1 DNA copy number in CD4$^+$ T lymphocytes could be a new indicator of the clinical status of HIV-1 infection in therapy-naïve patients. In addition, the delayed HIV-1 infection of CD4$^+$ T lymphocytes could provide new insights into anti-HIV-1 therapy. Selective therapy against M-tropic

A) HIV-1 DNA <DL



B) HIV-1 DNA >DL

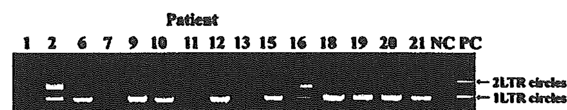


Fig. 2. Detection of unintegrated 1LTR and 2LTR circular HIV-1 DNA in 21 therapy-naïve patients. Molt4 cells were used as negative control (NC) and Molt4-IIIB cells persistently infected with HIV-1 IIIB were used as positive control (PC). Products of PCR electrophoresed in 1% agarose gels were visualized by staining with ethidium bromide.

Table 4. Detection rate of circular HIV-1 DNA in therapy-naïve patients classified as three categories

Categories	Detection rate of circular HIV-1 DNA
CD4$^+$ cell count (cells/µl)	
<200	4/4 (100%)
200–350	6/10 (60%)
>350	2/7 (28.6%)
Plasma HIV-1 RNA (copies/ml)	
<50,000	3/9 (33.3%)
50,000–100,000	2/5 (40.0%)
>100,000	7/7 (100%)
Intracellular HIV-1 DNA (copies/10^6 cells)	
<DL	1/6 (16.7%)
200–10,000	7/11 (63.6%)
>10,000	4/4 (100%)

HIV-1 might retard HIV-1 infection of CD4+ T lymphocytes, delaying or preventing subsequent formation of a lymphocytic HIV-1 reservoir.

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